ISOLATION AND IDENTIFICATION OF THE ABNORMAL OPAQUE MATERIAL IN THE WHITE MUTANT OF SIAMESE FIGHTING FISH *BETTA SPLENDENS*

An abstract of a Thesis by
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April 1976
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The problem. White purine pigments commonly occur as iridophore coloration in a variety of animals. These purines may also be abnormally stored in body integuments. A new mutation, opaque (Op), has been described for the Siamese Fighting Fish, *Betta splendens*. A build-up of the white material occurs as the fish ages especially in the head and eyes. Unlike a disease, the condition seems to be irreversible. The purpose of this research was to isolate and identify the constituents of the opaque white material.

Procedure. Any determination of purines in Op white Bettas must account for the guanine in iridophores. Three phenotypes, (i) Op, (ii) spread iridocytes (Si), and (iii) normal, were compared to distinguish between iridophore purines and the white material of the Op Bettas. A fourth phenotype, a hybrid (Op x Si), was included to determine possible differences in genetic expression.

The fish were microscopically inspected both externally and internally. Then various tissue samples were extracted (0.1N HCl) and components of the extracts separated by thin layer chromatography (1M NaCl). These separated components were then extracted (0.1N HCl) and the ultraviolet and fluorescent spectra were determined.

Findings. Microscopic inspection of the Op Bettas indicated iridophores were spread over the body and fins but not on the head as a result of the Si mutation. The white material covered the entire body and fins including the head and eyes. Thin layer chromatography, U.V., and fluorescence spectra indicated guanine was present in Op, Si, and hybrid Bettas. Comparison of guanine in head extracts showed 3.5 and 2 times more guanine in Op Bettas than in Si and hybrid Bettas, respectively.

Conclusions and Recommendations. The Op mutation may occur as a metabolic malfunction or as a purine-pteridine chromatophore inter-relationship. Guanine gout may result when a lack of xanthine dehydrogenase or guanase prevents

guanine destruction to normal by-products. Alternately, increased enzyme activity by guanine deaminase may produce excess guanine that is not normally catabolized and eliminated. A malfunction in chromatophore metabolish might also cause this mutation. An inverse relationship is observed in that purines increase in chromatophores when pteridines decrease. This may be either an enzymatic or hormonal disorder of the chromatophore. Enzymic and hormonal analyses should be done on the Op Bettas. Several genetic crosses are also needed with subsequent pigment, enzyme, and hormone analyses of the hybrids.

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A Thesis

Presented to

The School of Graduate Studies

Drake University

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

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by

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April 1976

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INTRODUCTION

White pigments commonly occur in a variety of animals. These normal white pigmenting materials are not harmful to the animals. A new mutation, Opaque, has been described for the Siamese Fighting Fish, Betta splendens. (1,2) A build-up of "chalk-white" or "creamy" material develops as the fish ages. The material spreads onto and, in some cases, over the eyes. In severe cases the eyes may become swollen as material deposits both inside and outside the cornea. This resembles the fish disease pop-eye and also suggests a glaucoma-like condition. However, fish with pop-eye can often be cured while the opaque abnormality seems to be irreversible. Breeding studies indicate the condition to be an inherited trait. (1,2)

Various animals, including man, store purines in the integument either normally or abnormally. If purines are found to be stored in the integument of Bettas with the opaque mutation, the animal may have an enzymic or hormonal malfunction affecting the interrelationship between various pigmentary materials. Gout, a condition in which purines are abnormally stored in body integument, is another possible cause of the opaque mutation. Further consideration of these depends on the isolation and identification of the constituents of the opaque material. Characterization of the material was the purpose of this research.

LITERATURE REVIEW

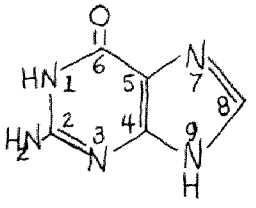
Pigmentation of plants and animals has stimulated the curiosity and esthetic sense of man since the dawn of civilization. It has often been wondered why organisms adapt certain colors and patterns and how these colors change. Although life forms are diverse, the chemical nature of pigments varies little from one organism to the next. (3) Four chromatophore types are generally recognized: melanophores, iridophores, xanthophores, and erythrophores. The occurrence and general characteristics of each are summarized in Table 1.

Table 1. Principle animal chromatophores.[a]

Chromatophore	Pigment	Color	Source
Melanophore	Melanins	Yellow, red brown, black	Invertebrates, vertebrates
Iridophore (Leucophore)	Guanine, adenine, hypoxanthine, uric acid	Reflecting platelets, or white	Vertebrates -- especially poikilotherms
Xanthophore	Pteridines -- especially sepiapterin; carotenoids	Yellow, orange, red	Poikilotherms
Erythrophore	Pteridines -- especially drosopterins; carotenoids	Red, orange, yellow	Poikilotherms

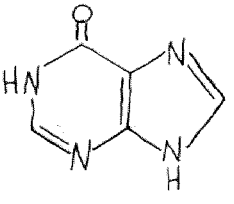
[a]Taken from Reference 3.

Iridophores and Purines in Fish. The metalic sheen and pearl essence of animals occurs when light is reflected from pigmented surfaces associated with iridophores. Among the purines responsible for the white or silvery tones of animals, guanine, 1, predominates. (3,4)

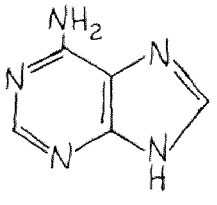


(2-amino-6-oxopurine) 1

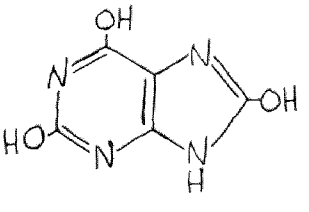
Guanine was identified as a constituent of fish scales by Barreswill and Bethe (5) in the late 1800's. Hitchings and Falco (5) and Neckel (6) formally demonstrated colorimetrically and microscopically the occurrence of guanine in fish integument. Although guanine is the primary constituent of iridophores (3,4), hypoxanthine, 2, adenine, 3, and uric acid, 4, have also been found. (3,5) Their structural similarities to guanine are apparent.



(6-oxopurine) 2



(6-aminopurine) 3



(2,6,8-trioxopurine) 4

These compounds are contained in relatively large granules or platelets that impart a birefringence to the chromatophore. Chemical analyses coupled with electron microscope studies reveal that free crystals of purine pigments exist in iridophores of fishes, amphibians and reptiles. (3,7-10) Studies on a variety of animals indicate that these reflecting platelets are highly variable in appearance, both between and within species. (11)

Denton and Land (12) showed that the iridophore cytoplasm of the scales of juvenile sprat, Clupea sprattus, consists of a stack of flat guanine platelets. Unpublished observations by Fujii (4) indicate granular guanine particles in the caudal leucophores of the goby, Chasmichthys. Guanine is the principle purine in both the Atlantic salmon, S. salar, (13) and juvenile Coho salmon, Oncorhynchus kisutch. (14) Guanine also occurs in the integument of cyprinid fishes, Squalius leuciscus and Alburnus lucidus (15), and the marine fish, Girella nigricans. (16) Large quantities have been extracted from the epidermis of scabbard fish. (17)

Hypoxanthine has been found with guanine in the belly skin of Coho salmon (14), the Atlantic salmon (13), and cyprinid fishes. (15) Uric acid has been described as the white material predominating in the hairtail, Trichirus maumela (18), and in minor amounts in the goby, Bathygobius. (19) Small amounts have been reported for other fishes. (20)

As with other animals, the brilliant green and metallic

blue colors of Bettas occur when iridophores are associated with other chromatophores. Mutations occur in Bettas resulting in changes in iridophore pigmentation. (21) These may cause an increase in the distribution of iridophores on the body and fins, and/or an apparent increase in the amount of pigmentary material in the iridophore.

APPROACH TO THE PROBLEM

Since various purines have been identified in fish integuments, these were focused on initially in this research. Qualitative identification of constituents of the opaque material is not sufficient since guanine is known to occur normally in the iridophores of Bettas. (21) Any determination of purines in the opaque white Bettas must account for the guanine in iridophores, particularly when the fish have the spread iridocyte mutation.

Various integument regions on the fish were used as sources for pigmentary materials. Integument samples were extracted and components of the extracts were separated by thin layer chromatography (TLC). The separated components were then extracted from the TLC plate and their ultraviolet and fluorescent spectra determined.

Materials were isolated from three phenotypes:[1]

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[1]Phenotype refers to the appearance of the animal in contrast to its genetic constitution.

opaque white--symbolized as Op, spread iridocytes--symbolized as Si, and normal. These were compared to distinguish between iridophore constituents and the creamy opaque material. A fourth phenotype, a hybrid, was included to determine possible differences in genetic expression of the Op factor between the homozygous[1] Op white and the heterozygous[2] hybrid. A table summarizing the genotype[3] for each subject is presented in Appendix A. The phenotypes used are depicted in Figure 1. The wild-type Betta from Thailand is the standard relative to which mutations are compared and described.

EXPERIMENTAL METHODS

Instruments. Ultraviolet spectra were obtained using a Beckman DK-2A. Fluorescence spectra were determined using an Aminco-Bowman 4-8202 recording spectrofluorimeter. A Cahn 1500, Model G, microbalance was used for weighing tissue samples.

Preparation of Standards. Analytical grade guanine and hypoxanthine were purchased from Fisher Scientific Co.

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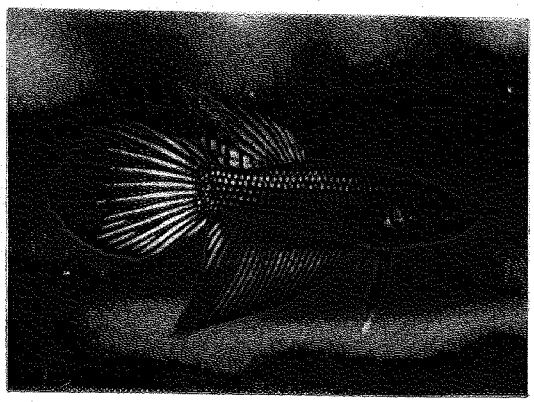
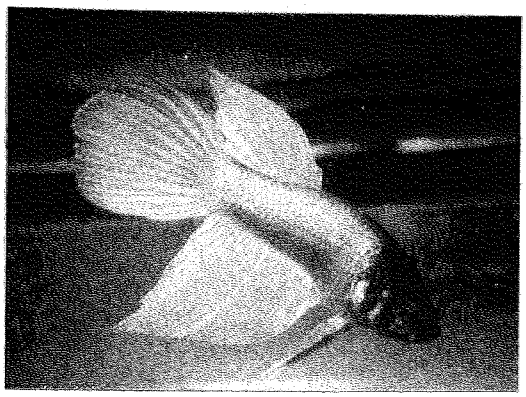
[1]Homozygous refers to the occurrance of two like genes at the same loci on homologous chromosomes.

[2]Heterozygous refers to a hybrid with two different genes at the same loci on homologous chromosomes.

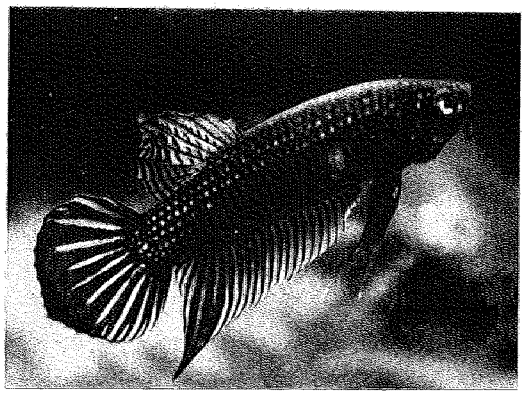
[3]Genotype refers to the genetic constitution of the animal.



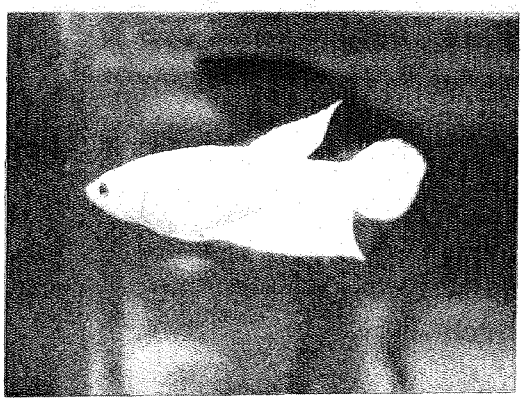
Normal Betta



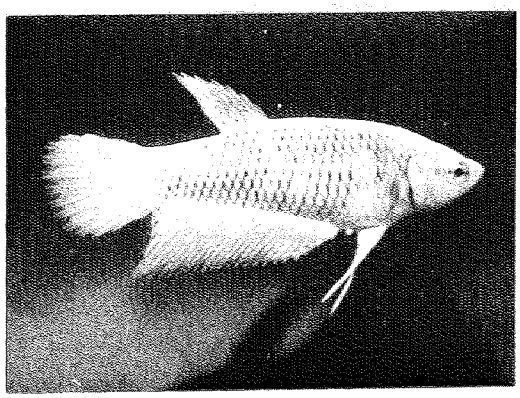
Spread Iridocytes Mutant



Wild-type, Betta splendens



Opaque White Mutant



Hybrid (Op x Si) Betta

Figure 1. Comparison of four phenotypes of Betta splendens.

(St. Louis, Missouri) and Aldrich Chemical Co., Inc. (Milwaukee, Wisconsin), respectively. Solutions (2×10^{-3} M) of each were prepared in 0.1 N HCl, and aliquots were used to make a standard guanine solution (3.00×10^{-5} M) and a standard hypoxanthine solution (3.60×10^{-5} M). Solutions were made to volume with temperature maintained constant at 25°C in a water bath. A hypoxanthine standard was also made in potassium hydrogen phthalate buffer solution, pH 7. This solution was used to compare absorbance and λ_{max} values with literature values.

Extraction and Component Separation. Fish were sacrificed in hot tap water (80°C). The integumentary regions used were: (i) fins including dorsal, caudal, and pelvic, (ii) body skin and pectoral fins, and (iii) head and eyes. Negligible amounts of underlying muscle tissue were removed.

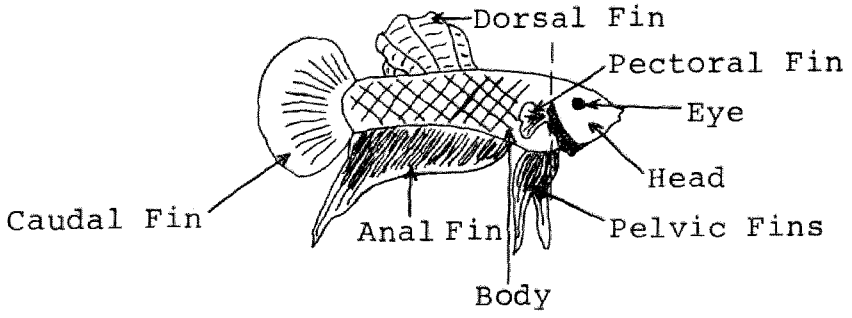


Figure 2. Integumentary regions used for purine extraction.

Integuments were extracted with 2 ml 0.1 N HCl (48 hr., ambient temperature). The extract was filtered through a sintered glass filter (fine porosity) into a 5 ml volumetric flask and diluted to volume with 0.1 N HCl. Standard

solutions and fish extracts were spotted (50 µl) on TLC plates (Eastman prepared cellulose with fluorescent indicator) and eluted with 1 M NaCl at ambient temperature. Plates were visualized with a short wavelength U.V. lamp. (22) The regions of the plates containing the separated components were scraped from the TLC plate, soaked in 2 ml 0.1 N HCl (48 hr., ambient temperature) filtered and diluted to volume as previously described.

Spectral Analysis. Solutions of components extracted from the TLC plates and standards were pipetted into Pyrex cuvettes (pathlength, 2 cm). Absorbance was recorded (320 to 225 nm) with the Beckman DK-2A; the reference was 0.1 N HCl. The fluorescence spectra of selected component solutions were also determined. The spectra were recorded as an emission scan with excitation at 248 nm, ratio recording; the reference was 0.1 N HCl.

Quantitative Methods. Integument samples were removed, dried in a dessicator over $CaCl_2$ (96 hr.) and then weighed on the Cahn microbalance. Components were extracted, separated by TLC and analyzed with the Beckman DK-2A as described above.

RESULTS

Standards. Chromatography of guanine and hypoxanthine standards gave non-fluorescing spots of R_f 0.33 and 0.56, respectively. They appeared as blue-grey shadows, neither

displaying any characteristic color.

Ultraviolet spectra of both unchromatogrammed and chromatogrammed guanine and hypoxanthine standards were characteristic for both with λ_{max} at 248 nm. The distinguishing spectral feature is the sholder present at 273 nm for guanine as seen in Figure 3. Absorbance values and λ_{max} are summarized in Table 2.

Table 2. Optical properties for unchromatogrammed guanine and hypoxanthine standard solutions, pH 1.

	Experimental Values			Literature Values			
	Guanine	Hypoxanthine		Guanine		Hypoxanthine	
λ_{max}	248 273(sh)	248	248.5(pH 7)	248[a]	248[b] 276(sh)	248[b]	250[a]
$\varepsilon(x10^{-3})$	11.4	11.1	13.8	11.4	11.4	10.8	10.6-10.9

[a]Taken from Reference 22.

[b]Taken from Reference 23.

Since the fish extracts were assumed to be rather dilute, the positions of λ_{max} with decreasing concentrations were examined. The results are shown in Table 3. Decreasing concentration shifts the observed λ_{max} to higher wavelengths. This also broadens and flattens the absorbance curve as shown in Figure 4.

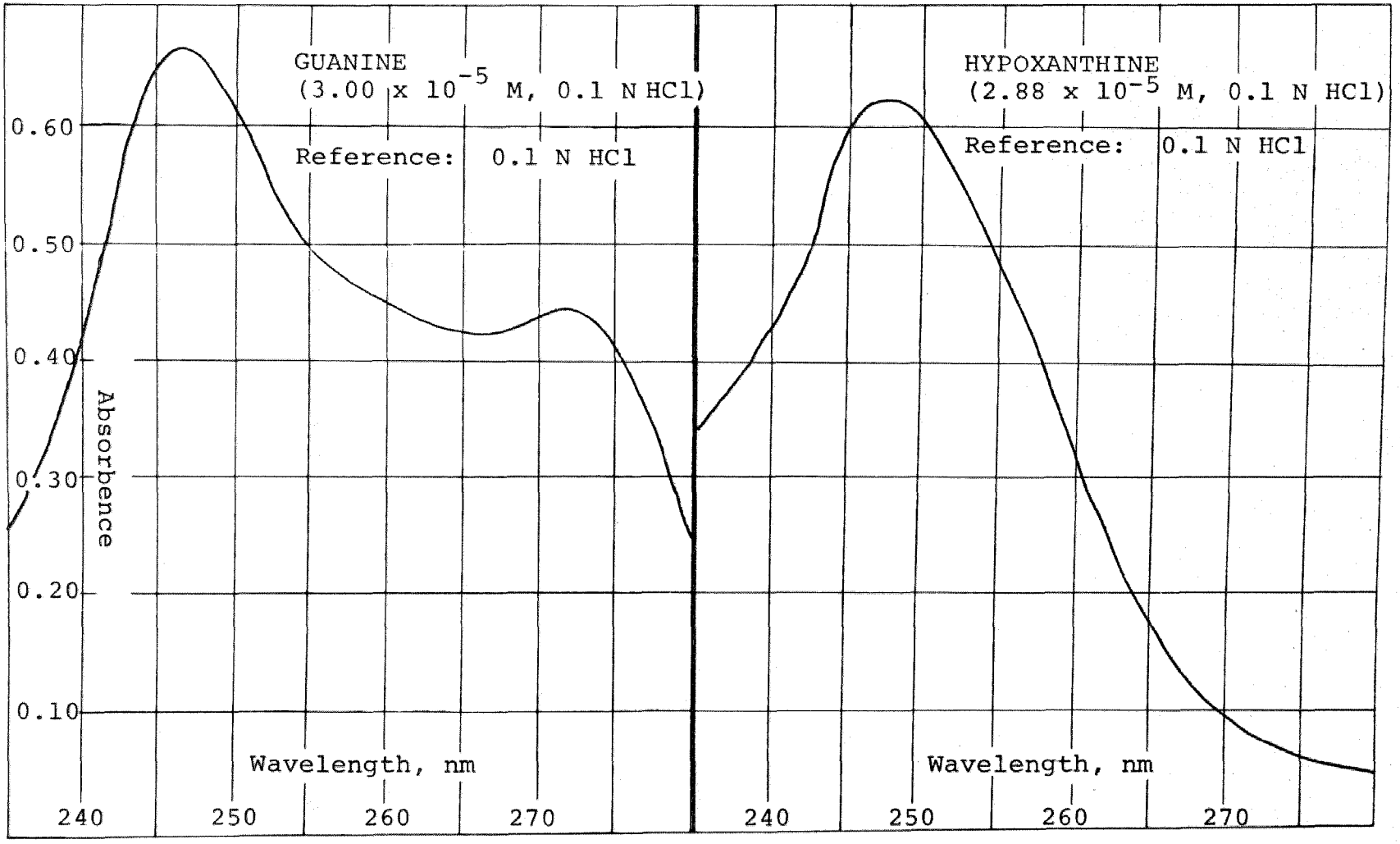


Figure 3. Ultraviolet spectra of guanine and hypoxanthine standards, unchromatogrammed.

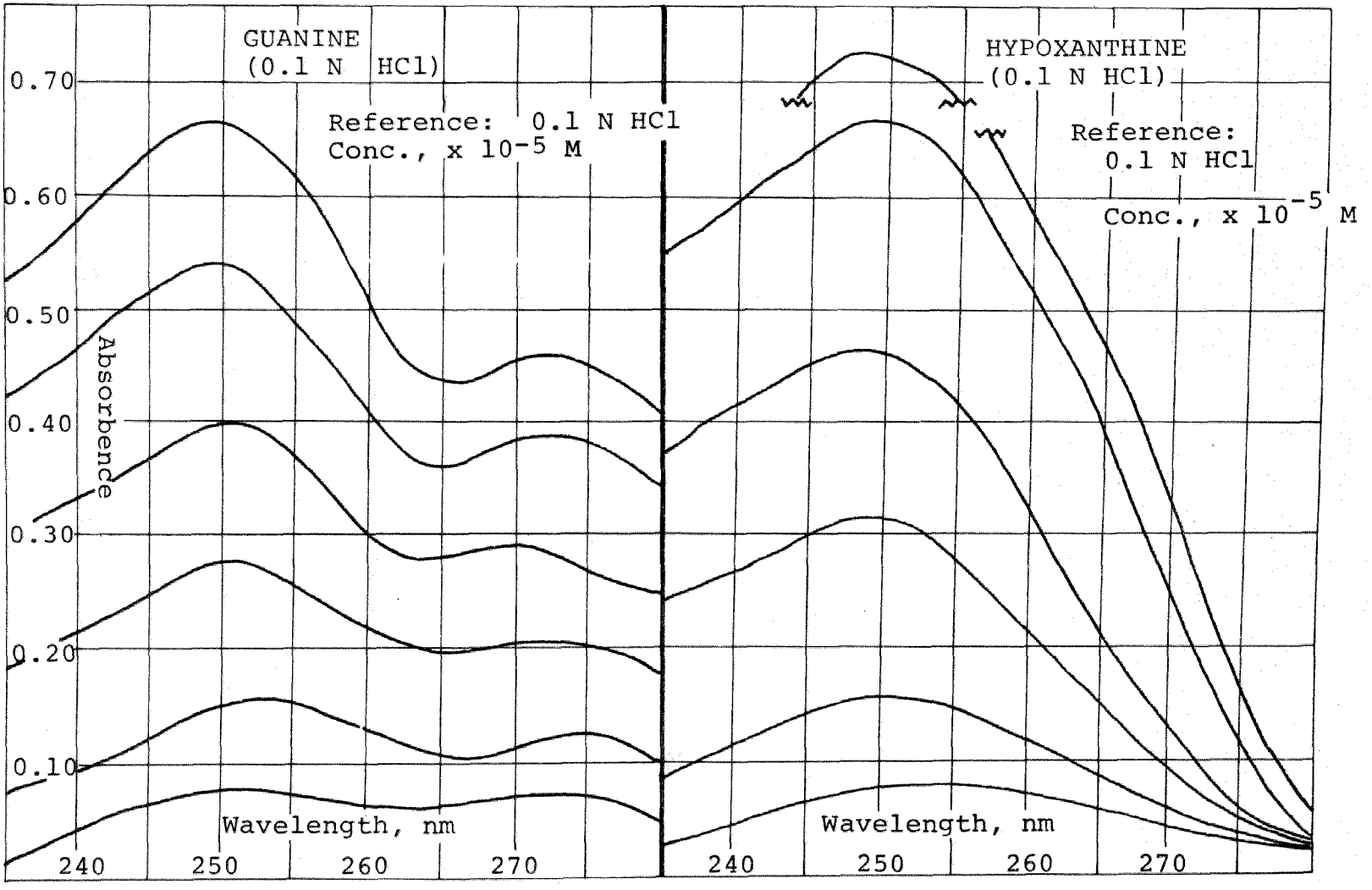


Figure 4. Ultraviolet spectra for decreasing concentrations of guanine and hypoxanthine standards.

Table 3. Effect of concentration on observed λ_{max} of guanine and hypoxanthine, pH 1.

	Concentration x 10^5	λ_{max}
Guanine	3.00	249
	2.40	250
	1.80	251
	1.20	252
	0.600	254
	0.300	255
Hypoxanthine	3.60	248
	2.88	249
	2.16	249
	1.44	250
	0.720	252
	0.360	257

Comparison of the fluorescent spectra of both unchromatogrammed and chromatogrammed guanine and hypoxanthine standards show the emission maxima changed only slightly when samples were chromatogrammed as indicated in Table 4. The emission peaks of guanine are 357 and 670 nm. No distinctive peaks were observed for hypoxanthine.

Table 4. Emission maxima and intensity from fluorescent spectra of guanine and hypoxanthine standards, unchromatogrammed and chromatogrammed.

Sample	Emission maxima, nm(Intensity)				
Solvent	257(12)	434(7)	498(58)		
Guanine - unchrom. (Sensitivity 0.3)	256(1.5)		495(8)	358(36)	679(5)
Guanine - chrom.	256(7)		498(50)	357(64.5)	670(10)
Hypoxanthine - unchrom.	255(4)	318-400(2)	496(16)		
Hypoxanthine - chrom.	256(6)	336-400(5)	496(30)		

Purines in Opaque White Bettas. Integument samples from six Op white Bettas were analyzed. Visual inspection of the subjects showed opaque material over the entire fins, body and head regions. Iridophores were in normal locations for fish with the Si mutation. (21) Microscopic inspection indicated iridophores were not located on the head nor were they on the webbing between the fin rays.

The eye had opaque material around the pupil in the iris. It was beginning to spread over the cornea as shown in Figure 5. When the skin of the anal fin webbing was tapped, glistening powdery white material was released. The scales of the body showed similar behavior. When dispersed

in the surrounding water, Brownian movement of the particles was observed suggesting that the opaque material was stored free in the integuments.

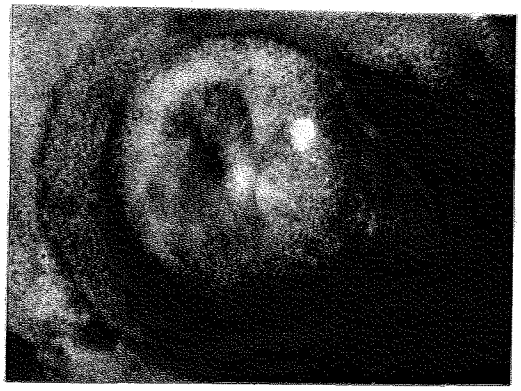


Figure 5. Eye of an opaque white Betta magnified 30x.

Dissection of the fish clearly showed opaque material deposited on the inside of the skin. Opaque-like material was also observed on the peritoneal membrane, although this is normal in small quantities.

Ultraviolet spectra were recorded for the integument extracts as a preliminary attempt to determine whether purines were present in the Op Bettas. Figure 6 indicates guanine was present in all three integumentary regions. Urea, uric acid, and allantoin were not indicated present (Appendix F).

Thin layer chromatography was used for separation of the components of fin, body, and head extracts (Appendix B). The results indicated both guanine and hypoxanthine were present in all three integumentary regions of the Op Bettas.

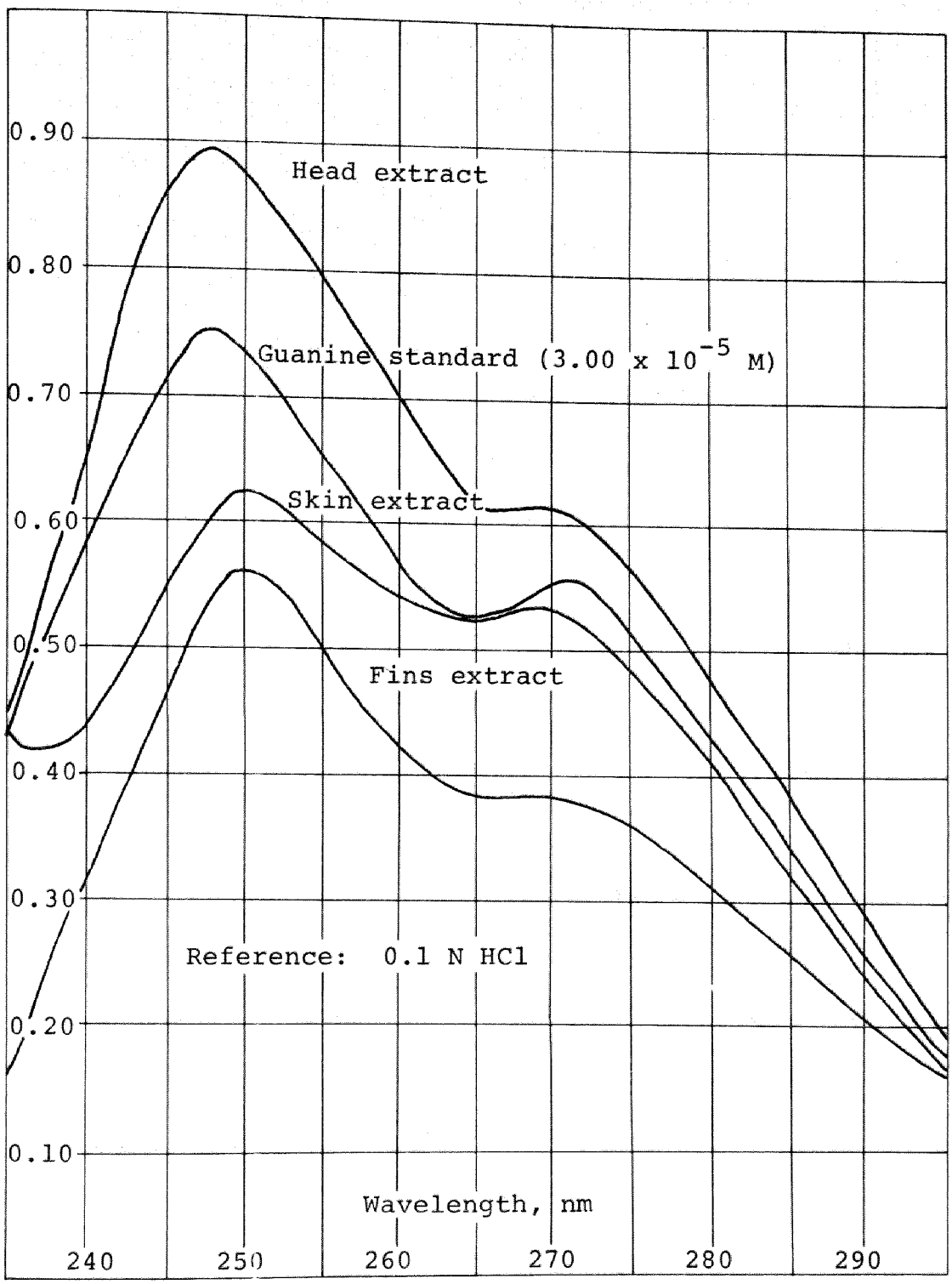
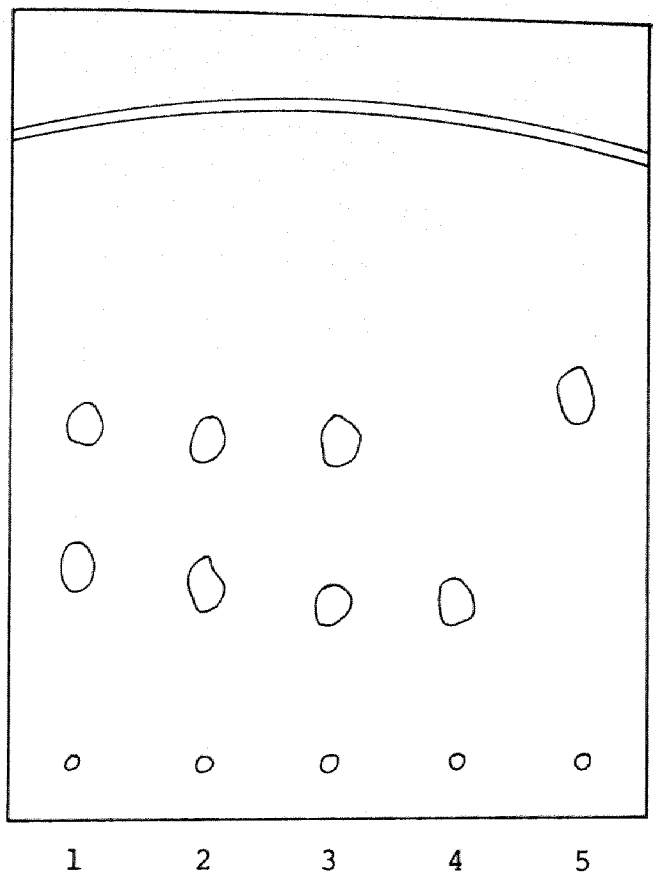


Figure 6. Ultraviolet spectra of integument extracts from opaque white Bettas, unchromatogrammed.

Figure 7 shows a typical TLC for the Op Bettas. The deviation between standards and samples may be caused by interference of pteridine pigments. (14) Presence of pteridine pigments was indicated by the green fluorescent color of the sample spots (R_f 0.00) and sample front when viewed under long wavelength U.V. radiation. (13,14)

Appendix C summarizes the U.V. spectra for integument extracts from the white fish. The average value of λ_{max} is 249 nm for guanine with the shoulder at 273 nm. The average λ_{max} for hypoxanthine is 250 nm. Figure 8 shows a typical guanine and hypoxanthine spectra for the fish integument extract. The average value of λ_{max} for the chromatogrammed guanine standards was 248 nm; that for the white fish integument extract was 249 nm. In all cases the shoulder at 273 nm was present.

Absorbance ratios for chromatogrammed guanine standard compared favorably with the literature values (Table 5). Ratios for material from fish are the same as the guanine standard except for the E250/E260 ratio in which E250 and E260 refer to the absorbance at 250 nm and 260 nm respectively. The discrepancy is probably because of pteridine interference. Incomplete pteridine separation during TLC would be most noticeable in the shorter wavelengths. This is supported by evidence from the U.V. spectra of pteridine materials; these showed end absorption from about 230 to 220 nm as indicated in Figure 9.



Chromatography System

Plate: Cellulose with fluorescent indicator (Eastman Prepared Chromagrams)
Solvent: 1 M NaCl, aqueous
Visualization: Short wavelength U.V. light
Temperature: Ambient

R_f Values

Guanine	Fins 0.29	Body 0.28	Head 0.29	Standard 0.33
Hypoxanthine	Fins 0.50	Body 0.48	Head 0.50	Standard 0.56

Figure 7. Separation by thin layer chromatography: typical chromatogram of integument extracts from opaque white Bettas.

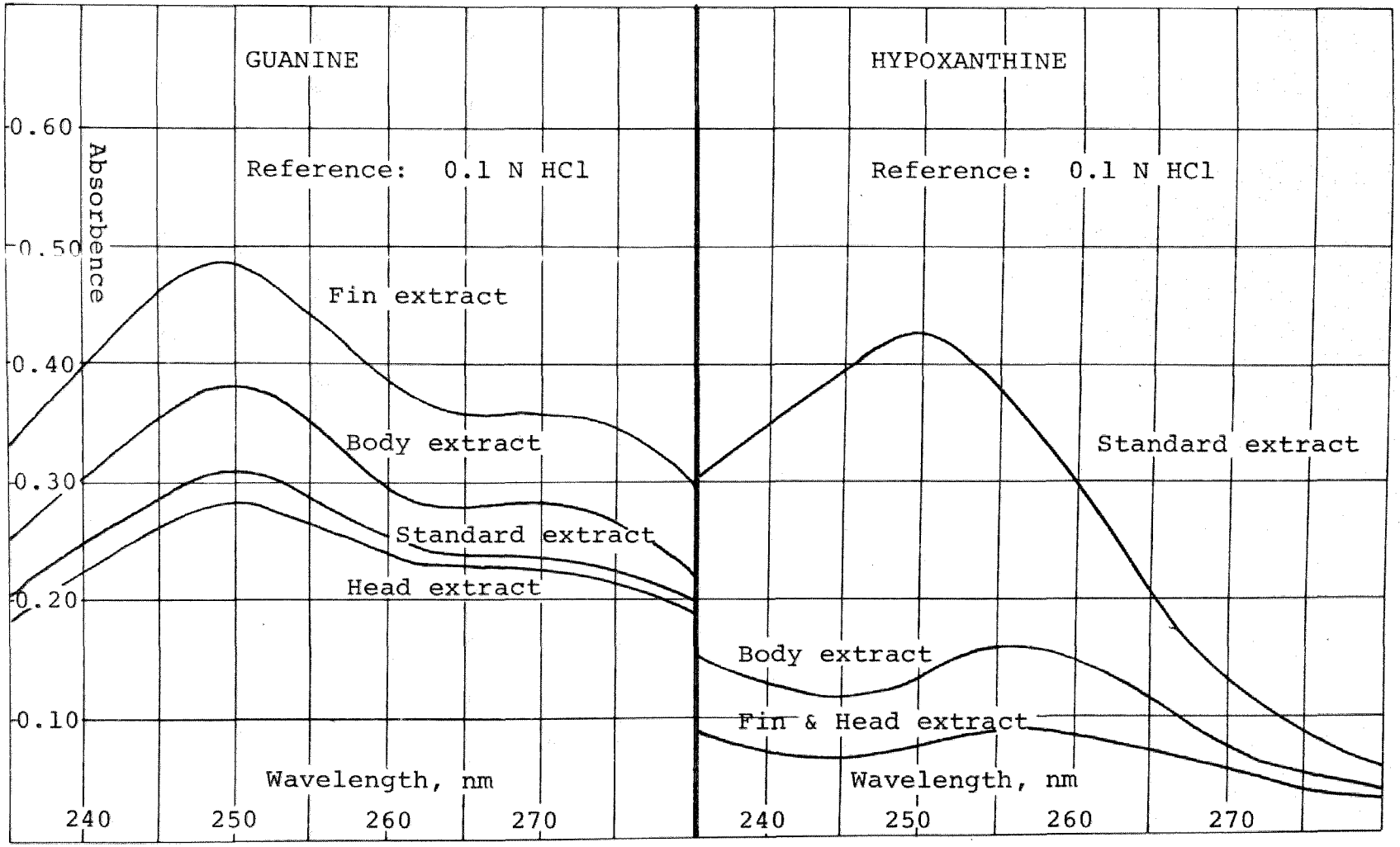


Figure 8. Typical ultraviolet spectra of guanine and hypoxanthine extracts from TLC of opaque white Betta integuments, chromatogrammed.

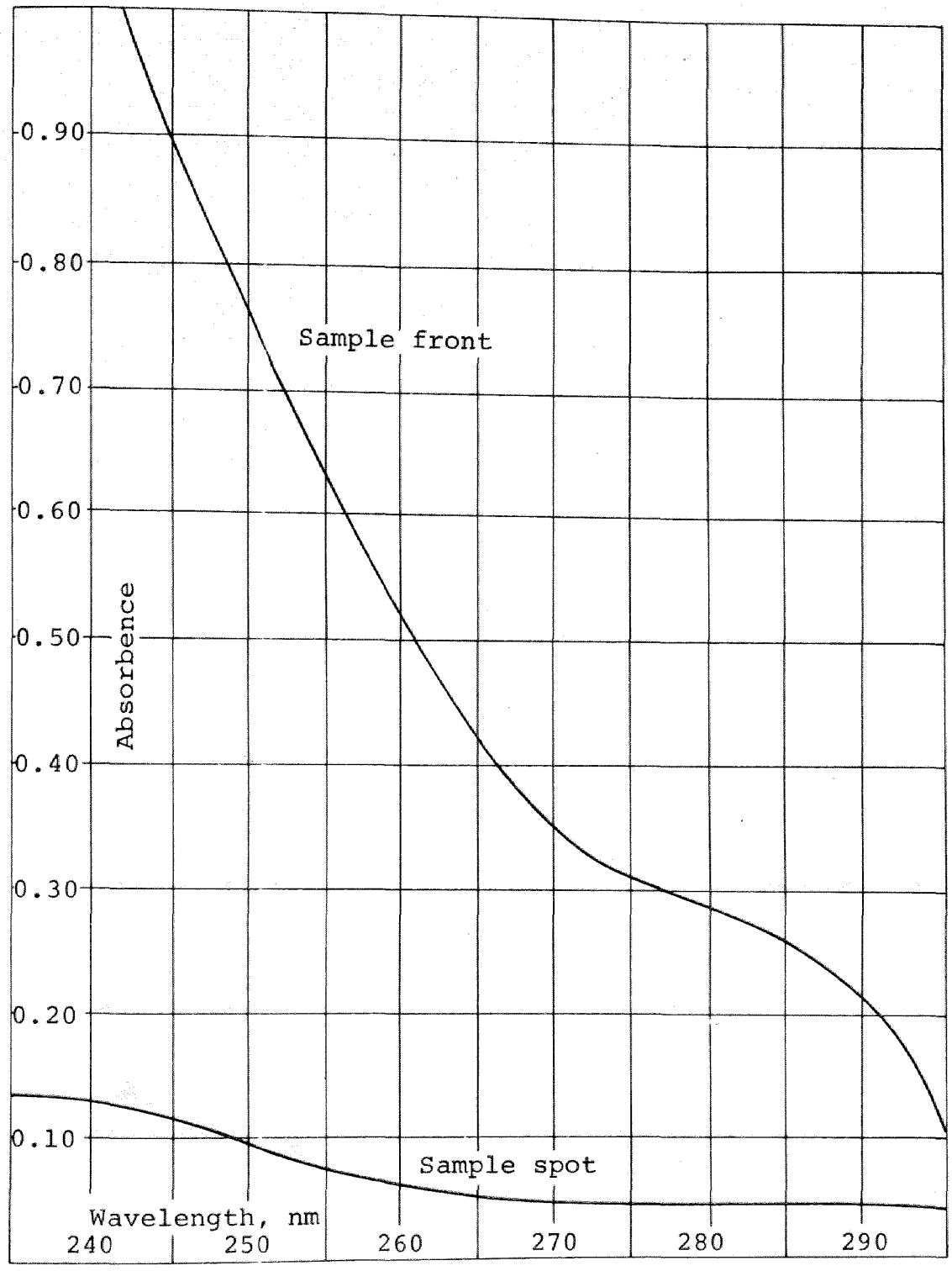


Figure 9. Typical ultraviolet spectra of pteridine pigments from opaque white Bettas, chromatogrammed.

Table 5. Comparison of absorbance ratios for guanine: literature values, chromatogrammed standard, and chromatogrammed fish extracts.

	E250/E260	E280/E260	E290/E260
Literature values[a]	1.37±0.03	0.84±0.02	0.48±0.02
Guanine standard, chromatogrammed	1.38	0.79	0.49
Fish extracts, chromatogrammed	1.27	0.79	0.48

[a]Taken from Reference 22.

The following ratios were also calculated for fish samples and standards: E250/E227.5 (max/min), E250/E270 (max/shoulder), and E270/E295 (shoulder/start). These values are given in Table 6. As expected, any pteridines not completely removed from the guanine spots show some end absorption and interfered most with the E250/E227.5 ratio.

Table 6. Absorbance ratios for guanine standard and guanine in opaque white Bettas.

	E250/E227.5	E250/E270	E270/E295
Guanine, standard	1.65	1.53	3.02
Guanine, Op Bettas	1.38	1.34	3.04

Fluorescent spectra (Appendix D) of guanine components from the Op Bettas gave the guanine peaks at 357 nm and 672 nm as indicated in Table 7. The intensity ratio

(357nm/670nm) was 6.4 for guanine standard and 6.0 for guanine from the fish.

Table 7. Emission maxima and intensity from fluorescent spectra of various purine components of opaque white Bettas.

Sample	Emission Maxima, nm (Intensity)				
Solvent	257(13)	434(7)	498(58)		
Guanine					
- chrom.	256(7)		498(50)	357(64.5)	670(10)
- unchrom. (Sensitivity 0.3)	256(1.5)		495(8)	358(36)	679(5)
Hypoxanthine - chrom.	256(6)	336-400(5)	496(30)		
Guanine - Op Bettas	257(10)		498(42)	357(60)	672(10)
Hypoxanthine - Op Bettas	256(12)	418(24)	498(68)		

Instrument: Aminco-Bowman 4-8202
Excitation: 248 nm
Emission scan
Ratio recording
Sensitivity: 1

Results of TLC, U.V., and fluorescence spectrophotometry indicate the principle purine present in opaque white Bettas in guanine. When compared to unchromatogrammed standards, the experimental values also show that the procedures used do not alter the purine. This suggests that free guanine is extracted from the fish rather than guanylic

acid or any other guanine derivative.

Further recovery and characterization techniques were not pursued. Based on percent recovery calculations (Figure 10), about 130 fish would be needed to collect 3 mg of impure guanine. This number of fish was not available. Had sufficient quantities of fish been available, NMR, IR, and elemental analysis would have been attempted. Thus, based on microscopic, chromatographic, U.V. and fluorescent spectra data, guanine was indicated as the principle purine pigment of the opaque white Bettas. The deviations between standard guanine samples and fish extract samples was probably caused by incomplete pteridine separation and subsequent interference. (23)

I. Percent Recovery - Guanine and Hypoxanthine Standards.

Guanine 76.7%

Hypoxanthine 84.9%

II. Total Guanine Isolated From Opaque White Bettas.

Based on 18 spectra:

93.6×10^{-8} mole/6 fish = 0.141 mg/6 fish

= 0.0236 mg/fish

For 1 mg: need 43 fish
For 3 mg: need 127 fish

Figure 10. Calculation of recovered guanine and hypoxanthine from standards and opaque white Bettas after thin layer chromatography.

Results were not as clear for the purine hypoxanthine. This component was suspected as a secondary purine pigment chiefly on the basis of chromatographic evidence. The typical guanine and hypoxanthine U.V. spectra shown in Figure 8 indicate that if hypoxanthine is present, its quantity, relative to that of guanine, is quite low. Using the average absorbance values for guanine and hypoxanthine from head extracts, the calculated concentrations (Equation 1) of guanine and hypoxanthine in the Op Bettas show about 50 times more guanine than hypoxanthine. Head extracts were selected for comparison since purines appear to be more concentrated in this region than in fin and body integuments (Table 8).

Equation 1. $$\text{concentration} = \frac{\text{absorbance}}{(\text{path length, cm})(\text{molar absorbance})}$$

$$c_{\text{guanine}} = \frac{0.324}{(2.0)(11.4 \times 10^{3})} = 1.47 \times 10^{-5}\ \text{M}$$

$$c_{\text{hypoxan.}} = \frac{0.007}{(2.0)(11.1 \times 10^{3})} = 3.2 \times 10^{-7}\ \text{M}$$

$$C_g/C_h = 46$$

Alternatively, the material may not be hypoxanthine. Since pteridines and purines have similar structures (1,5,6), and since pteridines are known to occur in these Bettas, this may be a non-fluorescing pterin. (24)

As indicated in Table 7 and as reported by others, the

fluorescence spectrum of hypoxanthine standard does not show a peak at the excitation wavelength used. (26) The peak at 418 nm for the fish extract hypoxanthine may be due to a pteridine impurity.

As with guanine, the results indicate that free hypoxanthine is extracted rather than a derivative. The evidence is, however, less conclusive for hypoxanthine since there is no characteristic property to be observed comparable to the 273 nm shoulder in the U.V. spectrum of guanine or the 357 and 670 nm fluorescent spectrum peaks.

Quantitative Determination of Guanine in Opaque White Bettas. Some guanine is present in the opaque white Bettas because of the spread iridocytes mutation (Appendix A). This mutation spreads iridophore color over the body and fins but not onto the head. A quantitative comparison of the spread iridocytes and opaque white mutants was done to determine whether the bulk of the guanine present in the opaque white Bettas is due to the opaque mutation or to merely the spread iridocytes mutation (Appendix E).

Comparison of the spread iridocytes mutant with the normal fish should show the relative amount of guanine present in Bettas because of the spread iridocytes mutation. The opaque whites may then be compared to the spread iridocytes mutant. Amounts of iridophore guanine should be about the same in both. Any qualitative variation or quantitative increase in purines may then be attributed to the

presence of purines in the opaque material in the Op mutant.

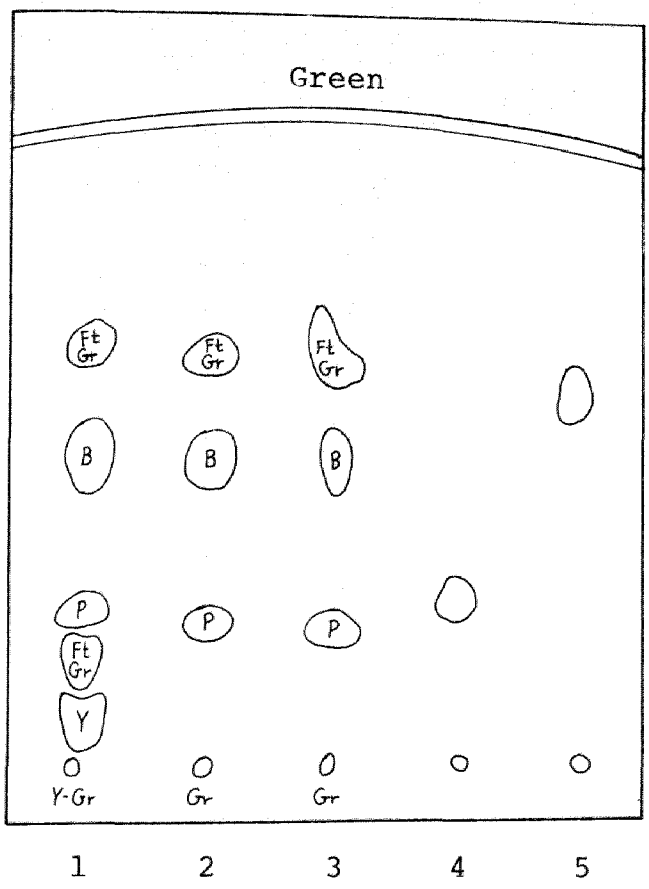
Both the normal and Si mutant had normal red as well as yellow pteridine pigments. These spots occurred with purine spots on TLC plates as seen in Figures 11 and 12. The average R_f value for guanine occurred at 0.33 for the normal Betta and 0.28 for the Si mutant. The R_f for the guanine standard was 0.33.

Variation between standard and fish extract R_f values was probably because pteridines were incompletely separated. Guanine spots from fish extracts with R_f 0.33 and 0.28 for normal and Si Bettas fluoresced under long wavelength U.V. radiation indicating pteridines rather than guanine.

The U.V. spectra of extracts from the normal Betta and Si mutant are given in Figures 13 and 14. Spectra of the normal Betta extracts indicate neither guanine nor hypoxanthine present. Guanine was detected in the Si mutant but hypoxanthine was not. Normalized absorbance values for guanine in the Si and Op mutants were calculated using the equation:

Equation 2. $$A_N = \frac{\text{Absorbance } (\lambda_{max}) \times 10^3}{\text{mg Integument in 5 ml solution}}$$

Results are tabulated in Table 8. These calculations show greater quantities of guanine in the Op Bettas, particularly in the head region. Thus, although guanine occurs as an iridophore pigment in Bettas, greater quantities occur as a



1 Fin extract

2 Body extract

3 Head extract

4 Guanine standard

5 Hypoxanthine standard

Fluorescence colors

Y yellow

Y-Gr yellow-green

Ft. Gr faint green

P purple

B blue

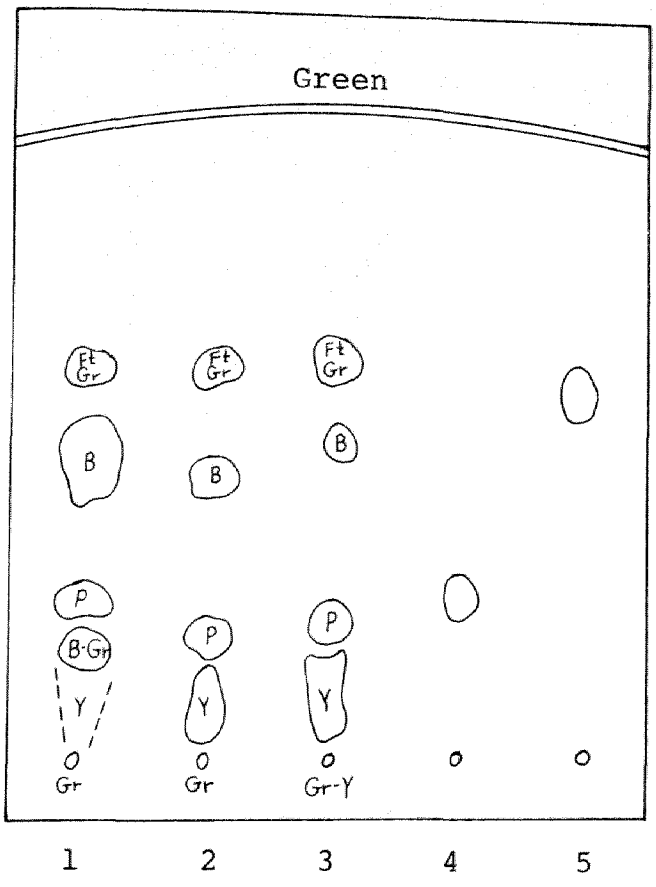
Chromatography System

Plate: Cellulose with fluorescent indicator (Eastman Prepared Chromagrams)
Solvent: 1 M NaCl, aqueous
Visualization: Short wavelength U.V. light
Temperature: Ambient

R_f Values

Guanine	Fins 0.32	Body 0.31	Head 0.35	Standard 0.33		
Hypoxanthine	Fins 0.52	Body 0.49	Head 0.52	Standard 0.56		
Pterins	0.00	0.09	0.18	0.29	0.50	0.64

Figure 11. Separation by thin layer chromatography: typical chromatogram of integument extracts from normal Bettas.



1 Fin extract

2 Body extract

3 Head extract

4 Guanine standard

5 Hypoxanthine standard

Fluorescence colors

Y yellow

Gr green

P purple

B blue

Gr-Y green-yellow

B-Gr blue-green

Ft. Gr faint green

Chromatography System

Plate: Cellulose with fluorescent indicator (Eastman Prepared Chromagrams)
Solvent: 1 M NaCl, aqueous
Visualization: Short wavelength U.V. light
Temperature: Ambient

R_f Values

Guanine	Fins 0.28	Body 0.28	Head 0.27	Standard 0.33			
Hypoxanthine	Fins 0.48	Body 0.57	Head 0.56	Standard 0.56			
Pterins	0.00	0.11	0.14	0.20	0.26	0.43	0.60

Figure 12. Separation by thin layer chromatography: typical chromatogram of integument extracts from spread iridocytes Bettas.

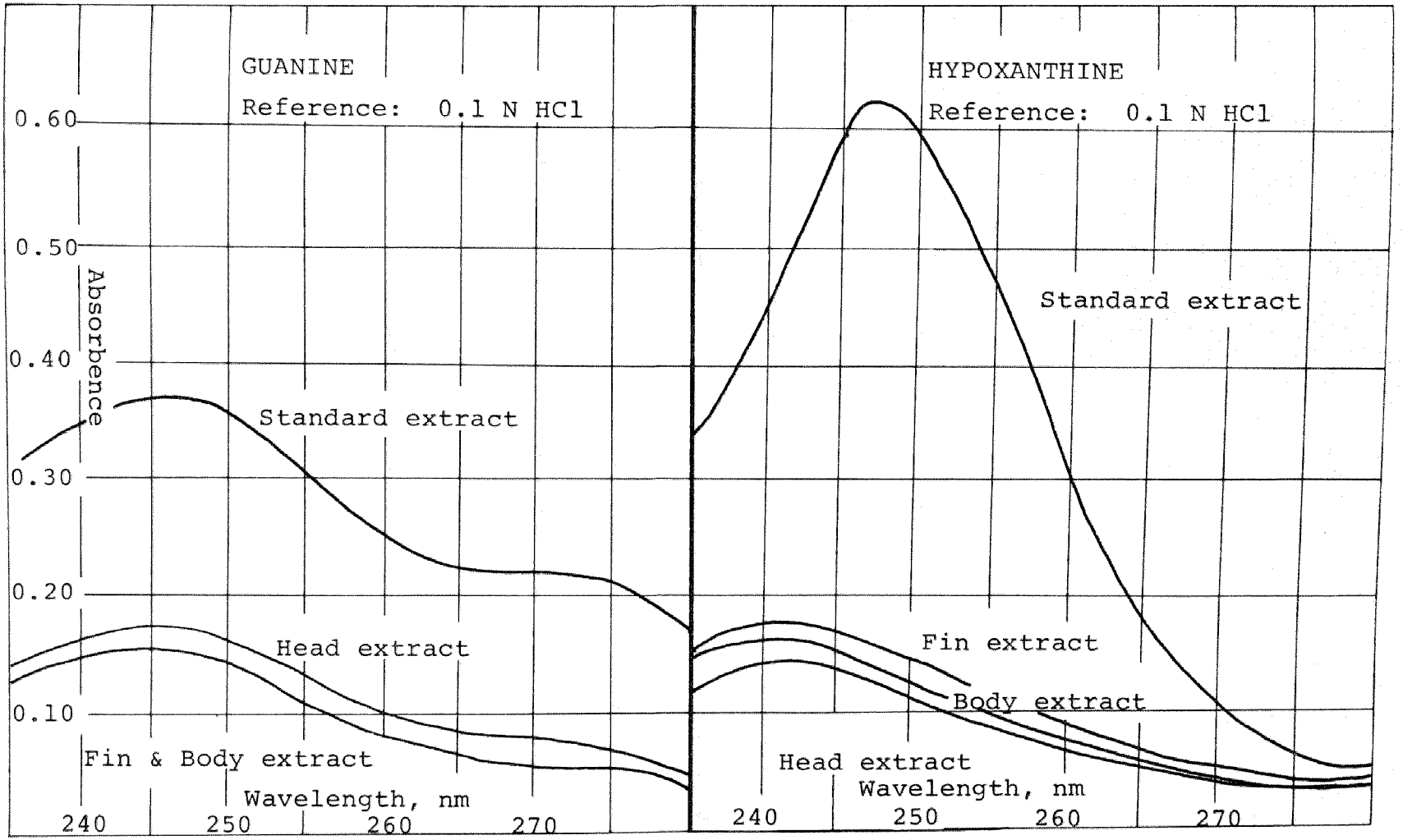


Figure 13. Typical ultraviolet spectra of guanine and hypoxanthine extracts from TLC of normal Betta integuments, chromatogrammed.

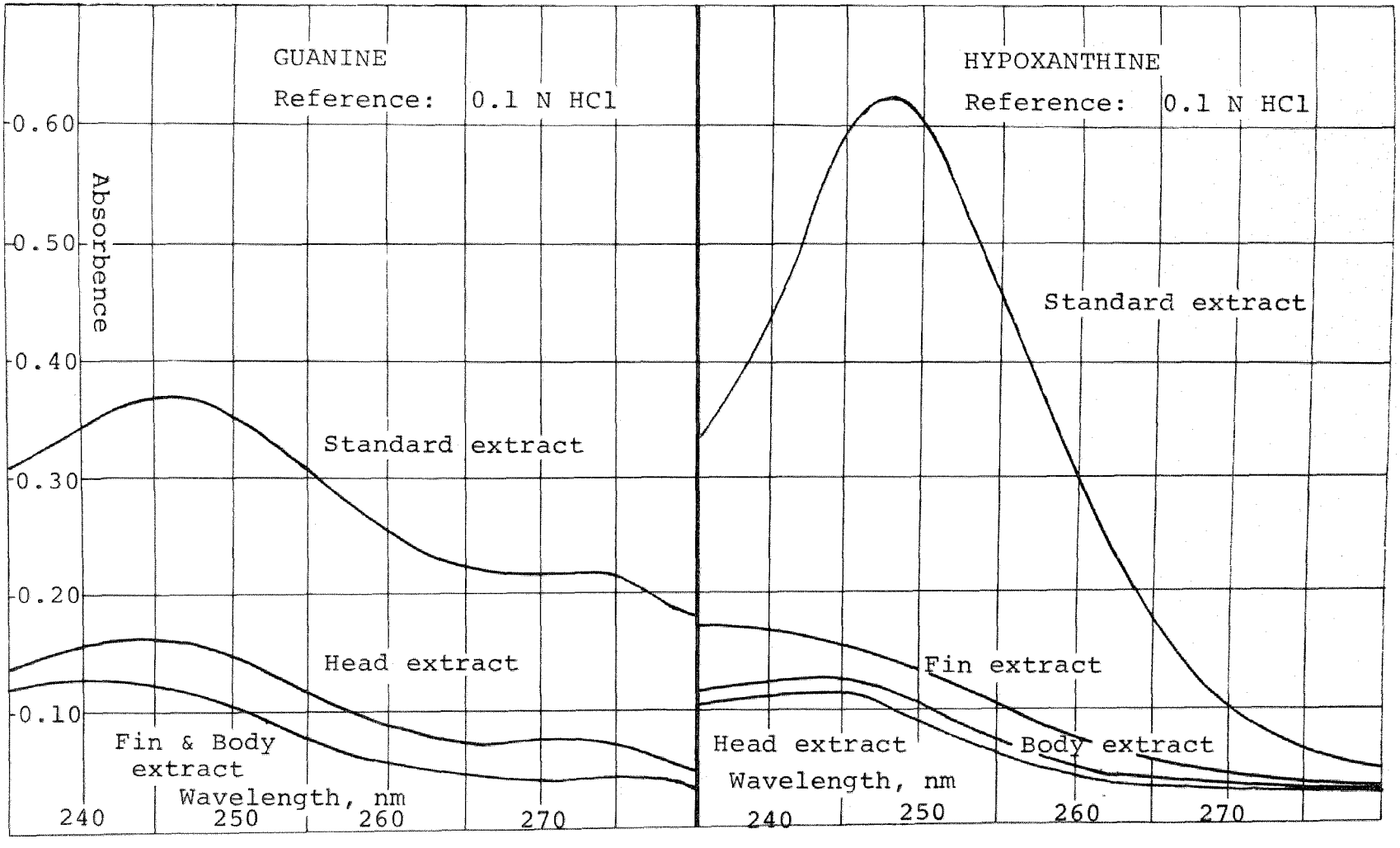


Figure 14. Typical ultraviolet spectra of guanine and hypoxanthine extracts from TLC of spread iridocytes Betta integuments, chromatogrammed.

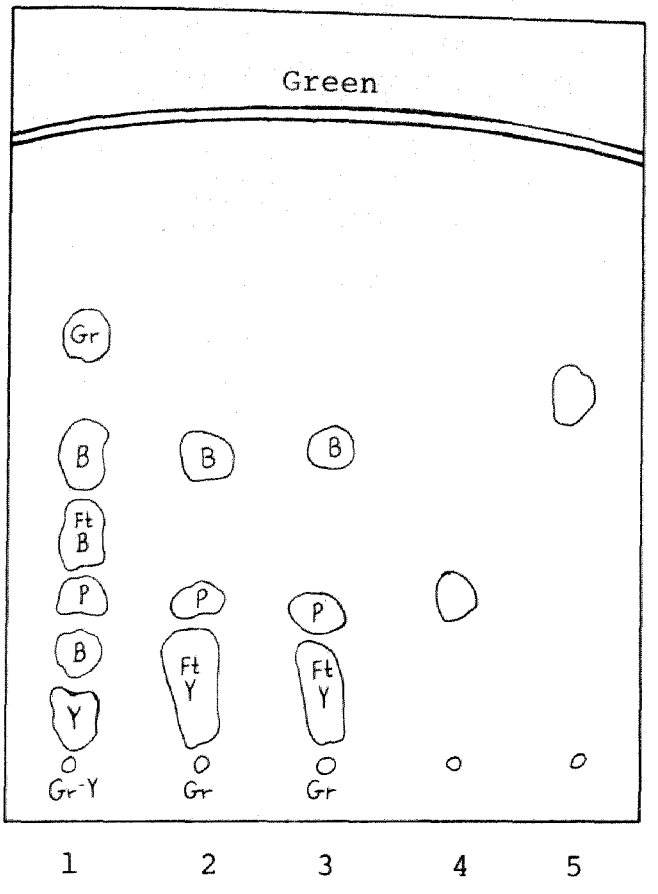
result of the opaque mutation, the material being spread over the entire body, especially the head.

Table 8. Comparison of A_N values for guanine in opaque white and spread iridocytes Betta mutants.

	Opaque White Bettas	Spread Iridocytes Bettas
Fins	6.85	4.24
Body	9.85	2.45
Head	61.50	9.15
Ratio of guanine in head extracts:	Op/Si = 6.72	

Quantitative Comparison of Opaque in Homozygous and Heterozygous Bettas. To obtain genetic inheritance information, the integuments of a hybrid fish (Appendix A) were also analyzed. The hybrid fish is an off-spring of an opaque white crossed with a spread iridocytes mutant. This hybrid is heterozygous and may be compared to the homozygous opaque white Betta. Quantitative changes in the amount of purine present might indicate the possibility of an incomplete dominance or intermediate form of the opaque mutation.

The hybrid Bettas had normal red and yellow pteridine pigments. These spots occurred with the purine spots on the TLC plates as seen in Figure 15. The guanine spot occurred at $R_f = 0.29$ and was visible with both long and short wavelength U.V. radiation. For the guanine standard, $R_f = 0.33$.



1 Fin extract

2 Body extract

3 Head extract

4 Guanine standard

5 Hypoxanthine standard

Fluorescence colors

Gr green

B blue

P purple

Y yellow

Ft. B faint blue

Ft. Y faint yellow

Gr-Y green-yellow

Chromatography System

Plate: Cellulose with fluorescent indicator (Eastman Prepared Chromagrams)
Solvent: 1 M NaCl, aqueous
Visualization: Short wavelength U.V. light
Temperature: Ambient

R_f Values

Guanine	Fins 0.29	Body 0.29	Head 0.30	Standard 0.33			
Hypoxanthine	Fins 0.49	Body 0.49	Head 0.52	Standard 0.56			
Pterins	0.00	0.14	0.21	0.32	0.39	0.51	0.64

Figure 15. Separation by thin layer chromatography: typical chromatogram of integument extracts from hybrid Bettas.

The U.V. spectra for hybrid Bettas are shown in Figure 16. The shape of the curves indicates guanine was present. Calculation of normalized absorbances for the hybrid Betta have values intermediate to the Si and Op mutants as shown in Table 9. The high value calculated for the fins of the hybrid probably reflects an incomplete pteridine separation. Again note the increase in guanine in the head region. Guanine ratios for the head extracts show the hybrid is 1.9 times greater than the Si mutant, and 3.5 times less than the opaque white.

Table 9. Calculated values of A_N for spread iridocytes, hybrid and opaque white Betta mutants.

	Opaque White	Hybrids	Spread Iridocytes
Fins	6.85	8.95	4.24
Body	9.85	5.10	2.45
Head	61.50	17.40	9.15

Guanine is also indicated in these hybrid Bettas by the fluorescence spectra (Table 10). The 356 and 678 nm peaks indicate guanine is present. The intensity ratio (356nm/678nm) for the hybrid Betta is 3.1 as compared to 6.4 for the guanine standard and 6.0 for the opaque white fish (Table 7). Several other peaks also occur in some of the fluorescence spectra of the hybrid extracts indicating an incomplete separation of purines and pteridines.

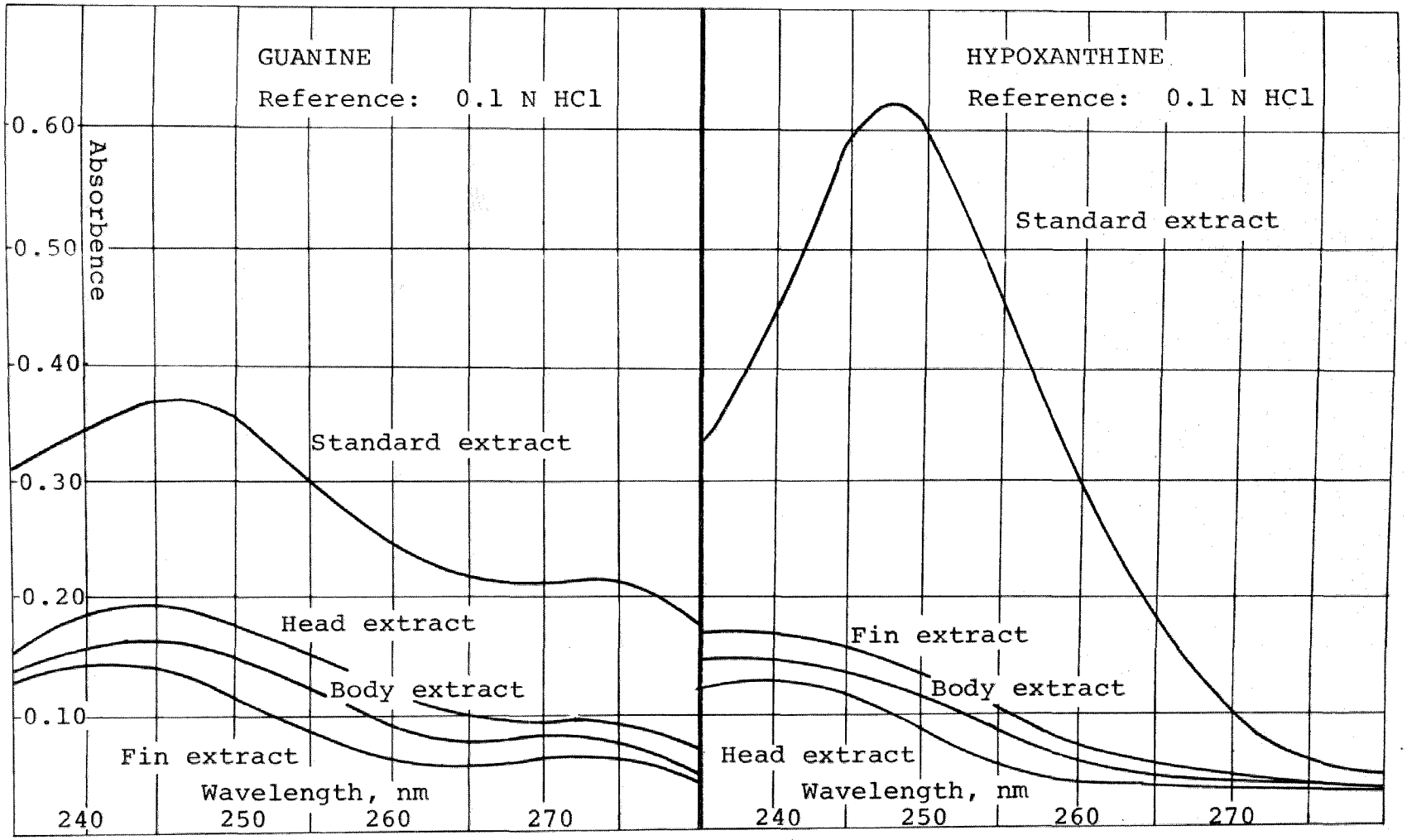


Figure 16. Typical ultraviolet spectra of guanine and hypoxanthine extracts from TLC of hybrid Betta integuments, chromatogrammed.

Table 10. Emission maxima and intensity from fluorescence spectra of guanine standard and purine components of hybrid Bettas.

	Emission Maxima (Intensity)				
Guanine, standard	256(7)	498(50)	357(64.5)		670(10)
Guanine, Hybrid Betta	256(11)	498(54)	356(38)		678(12)
			312(16)	348(19) 590(8)	

DISCUSSION

Summary of Results. Analysis of TLC, ultraviolet and fluorescence spectral data indicates guanine is the predominant purine producing the creamy white coloration in opaque white Bettas. Guanine also appears to be a constituent of the iridophores of Bettas as determined by comparison of TLC, U.V., and fluorescent spectra for the normal Betta and the Si mutant. Evidence was obtained for the presence of hypoxanthine or a non-fluorescing pteridine in the opaque white and hybrid Bettas. Spectroscopic analysis of fish extracts indicated urea, uric acid, and allantoin were not present. Thin layer chromatography spots other than guanine and possible hypoxanthine were fluorescent purple, blue, or green under long wavelength U.V. radiation suggesting they might be pteridines. (13,14,26) Since pteridines probably do not contribute to white pigmentation they were not

analyzed further. (14,27,28)

Quantitative comparison shows greater amounts of guanine present in the homozygous opaque white than in the heterozygous hybrid Bettas. This supports the hypothesis that the opaque factor is genetically inherited, possibly as a single gene action. (29) Guanine was indicated present, in decreasing amounts, for the opaque white, hybrid, and spread iridocytes Bettas. The U.V. spectra showed negligible guanine for the normal Betta which had minimal iridophore coloration and lacked the Op mutation.

For the extraction method selected, there existed the possibility of nucleoside and nucleotide degradation. Purines are more soluble in acid than in neutral or alkaline systems. Hydrolysis would, however, free the constituent nucleoside or nucleotide bases. The U.V. spectra of the purines in the Op Bettas indicated the principle component was guanine and not guanosine or a substituted derivative of either guanine or guanosine since substitution greatly changes the position of λ_{max}, the shoulder, and the absorbtivity. Examples of these shifts are shown in Table 11.

Table 11. Changes in the ultraviolet spectrum of guanine with substitution, pH 1.[a]

	λ_{max}, nm	Shoulder, nm	$\varepsilon_{\lambda_{max}} \times 10^{-3}$
Guanine	248	276	11.4
1-Methyl	250	272	10.2
N^2-Methyl	250	279	6.2
N^2,N^2-Dimethyl	256	250	19.0
7-Methyl	272		6.9
-Br (at C-8)	259	256	21.2
$-N(CH_3)_2$ (at C-8)	255	288	20.1
$-S-CH_3$ (at C-8)	273	290	13.8
Guanosine	256		12.3
1-Methyl	258		9.4
N^2-Methyl	258		14.3
N^2,N^2-Dimethyl	264		12.8
7-Methyl	257		10.7
2'-O-Methyl	256		10.7

[a]Taken from Reference 31,32.

Guanine and 1-methylguanine appear quite similar in their U.V. spectra. However, they may be distinguished by fluorescence spectrophotometry (Table 12). Udenfriend (30) and Undenfriend and Zaltzman (25) have reported the use of fluorescence spectrophotometry in the identification of purines, pyrimidines, and their derivatives. While some of

the purines and their derivatives, such as guanine, its nucleotides, nucleosides, and some of its methylated derivatives, are intensely fluorescent compounds when excited at their absorption maxima, others, including hypoxanthine, are extremely low. The fluorescence spectra obtained in this research indicate guanine is present. The 357 nm maximum of standard and fish extracted guanine closely approximates the 360 nm maximum reported in the literature for guanine rather than the 370 nm maximum of 1-methylguanine. Neither hypoxanthine standard nor the fish extracted component gave a fluorescence spectrum.

Table 12. Fluorescence characteristics of guanine, 1-methylguanine, and hypoxanthine, pH 1.

Compound	Absorption max., mμ	Excitation max., nm	Fluorescence max., nm	Relative intensity
Guanine[a]	272 (sh)	285	360	---
Guanine[b]	272 (sh)	275[c]	360	200
1-Methyl-guanine[b]	274 (sh)	290	370	150
Guanine, standard,	273 (sh)	248	357	64.5
Guanine, Op Bettas	273 (sh)	248	357	60
Hypoxan-thine[b]	---	---	---	0
Hypoxanthine, standard	248	248	negligible	0
Hypoxanthine, Op Bettas	248	248	negligible	0

[a]Taken from Reference 30.

[b]Taken from Reference 25.

[c]Excitation spectra corrected as described in Reference 25.

Standard solutions of guanine and 1-methylguanine were subjected to TLC to further distinguish between them. However, the results were not conclusive because of the impure nature of the commercially obtained 1-methylguanine. As shown in Appendix H, the 1-methylguanine gave two component spots. This occurred when two different systems were used. Double concentrations of 1-methylguanine were run with and compared to guanine in an attempt to observe the relative concentrations (via spot darkness) of the separated 1-methylguanine components. Guanine, the parent compound, appeared to be one of the major components of the 1-methylguanine standard. Another major spot occurred at R_f = 0.60 and may be the 1-methylguanine component (Appendix H, plate 1). A minor, faint spot also occurred at R_f = 0.44.

Other runs in which the standards were run separately and in combination with fish extracted guanine indicated the fish material was indeed guanine rather than 1-methylguanine. Only a single spot was observed with an R_f value corresponding to that of guanine when guanine and fish extract material were run mixed. Two spots, a guanine (R_f = 0.37) and a second component spot appeared when fish extract was mixed with either hypoxanthine or 1-methylguanine (Appendix H, plates 2,3).

The originally separated material from the Op fish gave two spots, a guanine component and a second component assumed to be either hypoxanthine or a non-fluorescing

pteridine. This second material was not 1-methylguanine. The U.V. spectra of this material did not give a spectra with the 273 nm shoulder characteristic of 1-methylguanine.

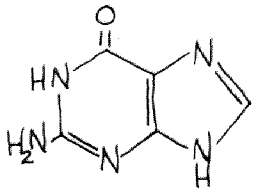
Possible Causes of Guanine Accumulation. Systems of color and color change existing among fish show great diversity. There are several possible explanations for purine accumulation in Bettas. Although environmental factors such as water temperature, light, and salinity affect guanine levels in some fishes such as salmon (4,13,14), these factors probably do not cause the increased guanine concentrations that are found in fish with the Op condition. These fish were housed under conditions of temperature and light identical to those of several other Betta color mutants none of which showed any accumulation of the "creamy" material. (33) The Op condition does not appear to be environmentally induced, but probably occurs as a genetic mutation.

The Op mutation, thus far, occurs only in fish where the Si mutation is also present. Crosses are needed to determine if Op can occur independently or if it is linked to the Si mutation, and if so, how. The Op factor first appeared in a green-gold stock where the creamy opaque substance mixed with the normal green color to give the gold-like appearance somewhat characteristic of the opaque material. (1) This indicates that both yellow and opaque pigmentary materials are present and seen through iridophore

coloration.

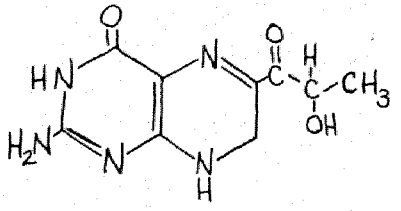
Opaque does occur in Bettas other than the white and pastel mutants. The effect is clearly seen in the hybrid stock used in this study. It is also observed in a red-head stock. Interestingly, the red of these fish does not appear as a bright or brilliant red typical of red Betta stocks. (29) It is somewhat dulled and off-color. Possibly it is a carotenoid red (26), or the dullness may be related to the Op mutation and a concurrent conversion and/or reduction of normal red pteridine pigments.

Interrelationship of purines and pteridines. It is only within the past 5 to 10 years that the complex interrelationship between the various chromatophores has been recognized. Chromatophores of fish are derived from the neural crest during their early development. (4) Among poikilotherms,[1] purines and pteridines are important pigments that differ markedly from one another functionally. Chemically, both contain two rings, one of which is a pyrimidine ring. The second ring in purines is a five-membered imidazole ring (5). This is replaced by a six-membered pyrazine ring in pteridines (6).



Guanine 5

[1]Poikilotherms are animals that tend to take on a body temperature similar to their surroundings. They include fish, reptiles, and amphibians.

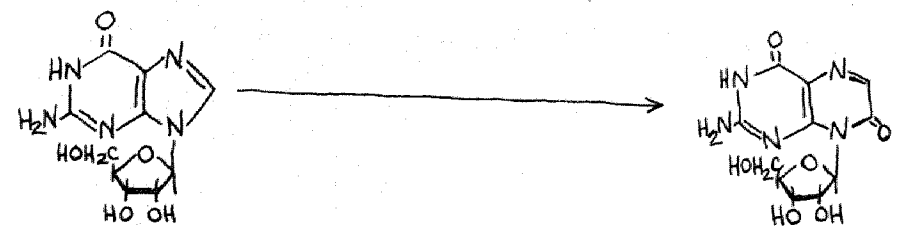


Sepiapterin (Yellow pteridine) 6

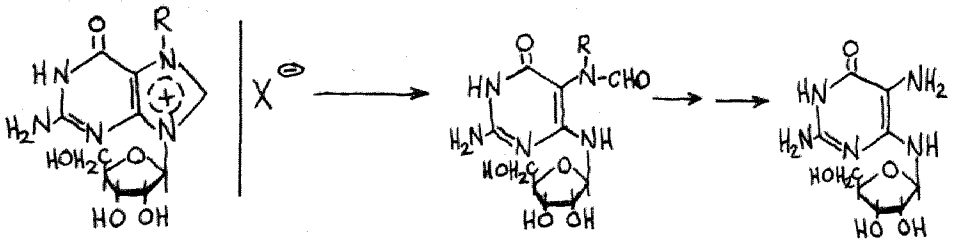
Chemical conversion of purines to pteridines. While the biosynthesis of purines is understood, much less is known about pteridine synthesis although evidence is accumulating that pteridines are synthesized through a purine precursor. (34,35,36) The first data which supported the contention that purines are precursors of pteridines was supplied by Reynolds and Brown in the early 1960's. (37) They showed that in extracts of E. coli, guanosine or guanine nucleotide could be converted to the pteridine portion of folic acid, with removal of the C-8 of the purine ring. They also found no other purine, purine nucleoside, or nucleotide could be utilized in place of guanine compounds.

Plfeiderer et al. (36) have chemically converted, as shown in Scheme 1, guanosine (I) into isoxanthopterin N_8-riboside (II). This reaction is of interest because most naturally-occurring pteridine derivatives originate from pterin (2-amino-4-pteridone). It involves cleavage of the imidazole ring in I. Though stable to acid or base hydrolysis, quaternization at N_7 (III) allows mild nucleophilic attack of a hydroxyl ion at position 8 to give the ring opening shown as IV ($R=CH_3$). As reported, further conversion of IV into V and II could not be achieved since all attempts

to remove the formyl group led to loss of the ribose residue.



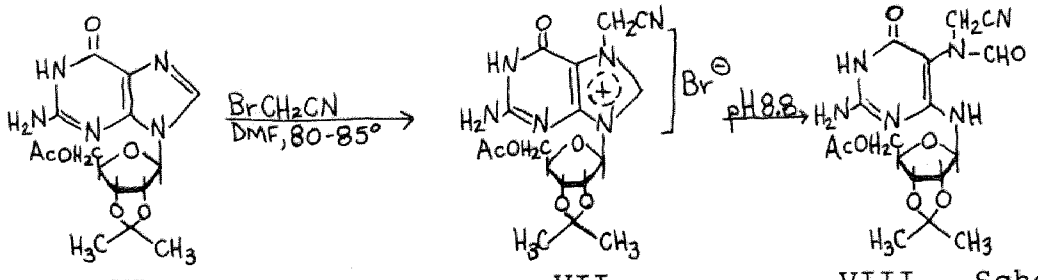
I II



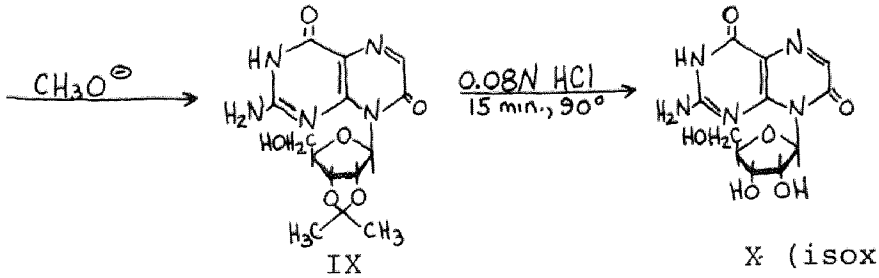
III IV V

Scheme 1

Pfleiderer et al. (36) reported successful conversion of guanosine to isoxanthopterin N_8-riboside (Scheme 2, X) by acylation of the unprotected 5'-OH group (VI) to stabilize the guaninium salt. This is shown in Scheme 2.



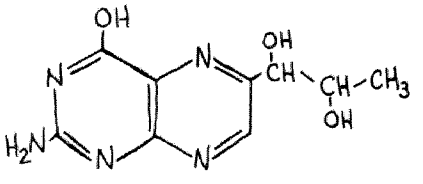
VI VII VIII



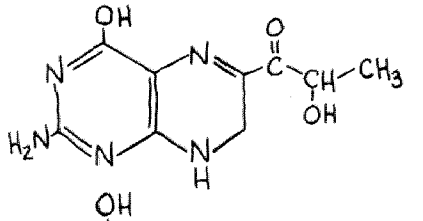
IX X (isoxanthopterin N_8-riboside)

Scheme 2

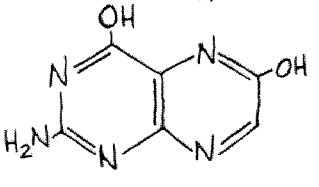
Biological conversion of purines to pteridines. While there are several naturally occurring pteridines, most are 2-amino-4-hydroxypteridines which may be substituted at the 6 position. The best known are biopterin (7), sepiapteridine (8), and xanthopterin (9), all of which occur as animal pigments. (3,38) Several lines of evidence indicate these unconjugated pteridines are made biosynthetically from guanine compounds. However, since no enzyme work has yet been done, not much is known of the details of the biosynthesis of these materials. (37)



Biopterin 7



Sepiapteridine 8



Xanthopterin 9

Greenburg (37) has reviewed the biogenesis of pteridines and the evidence that they are formed from purine precursors. Waygand and Woldschmidt (37) provided the first biological evidence that purines and pteridines are related. Butterfly larvae were injected with various compounds labeled with ^{14}C. The isolated pteridines, leucopterin (white

pteridine) and xanthopterin (yellow pteridine), were isolated and the labeling pattern compared to purines. They found that glycine and formate were incorporated into similar positions as shown in Figure 17. Other studies substantiate these findings. (39,40,41)

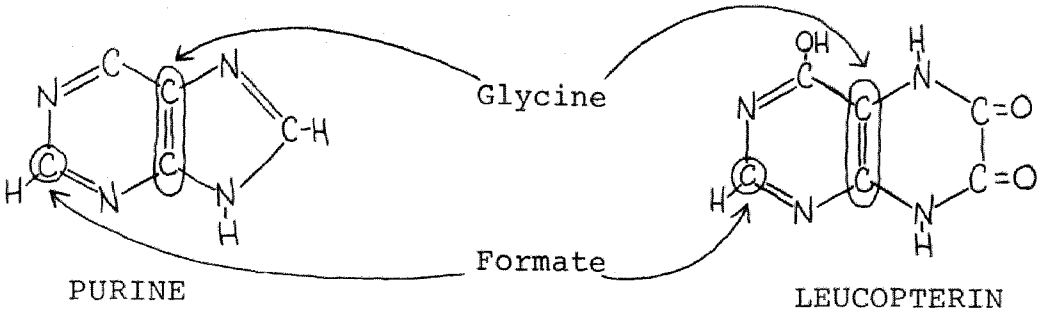
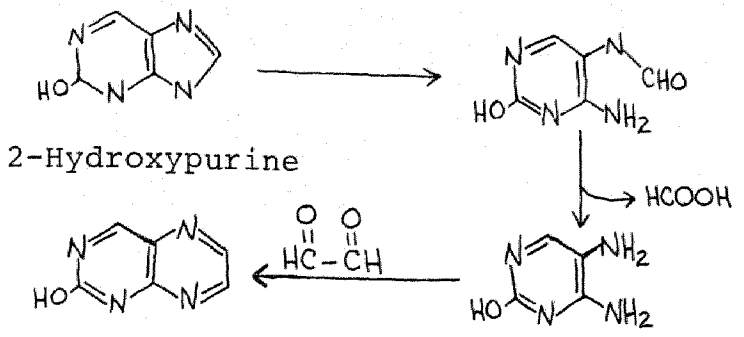


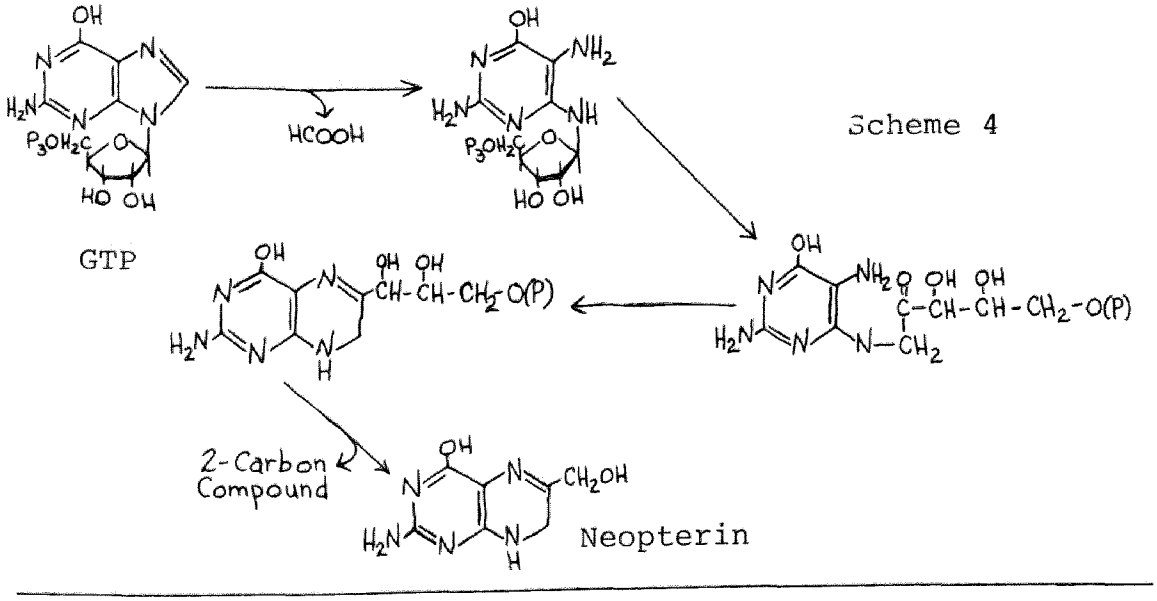
Figure 17. Incorporation of glycine and formate into purines and pteridines.

Since Albert's (42,43) first clue as to the origin of pteridines via a purine precursor, as shown in Scheme 3, the question arose how pteridines might biosynthetically be made from purines; i.e., what precursor(s) of C-6 and C-7, provided by glyoxal in Scheme 3, occur in nature? *In vivo* ^{14}C incorporation experiments indicate that these carbons are efficiently supplied by glucose or ribose in butterflies, the fruit fly (*D. melanogaster*), and bacteria. (37)



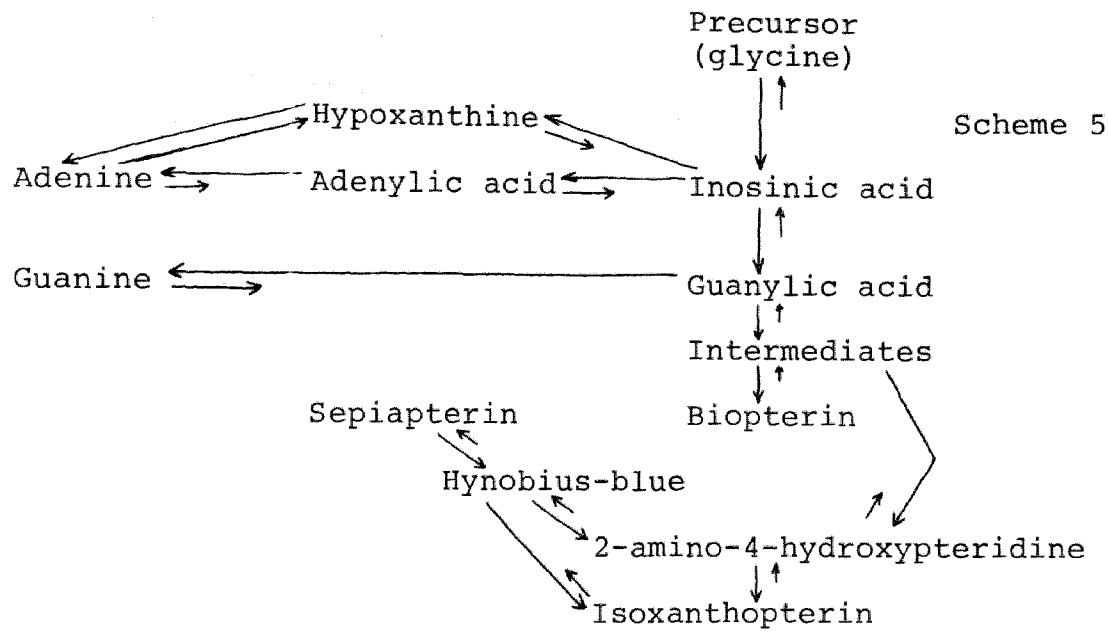
Scheme 3

Subsequent investigations, as reviewed by Greenburg (37), support the notion that purines are direct precursors of pteridines. The suggested reaction sequence is shown in Scheme 4. Guanosine-5'-triphosphate (GTP) is the only biologically active substrate that may be used in this reaction sequence.



Scheme 4

Stackhouse (27) has proposed a scheme relating pteridine and purine biosynthesis as indicated in Scheme 5. This scheme is postulated for the biosynthesis of pteridines and their relationship to purines in hypophysioprivic[1] frog larvae, Rana sylvatica.



Scheme 5

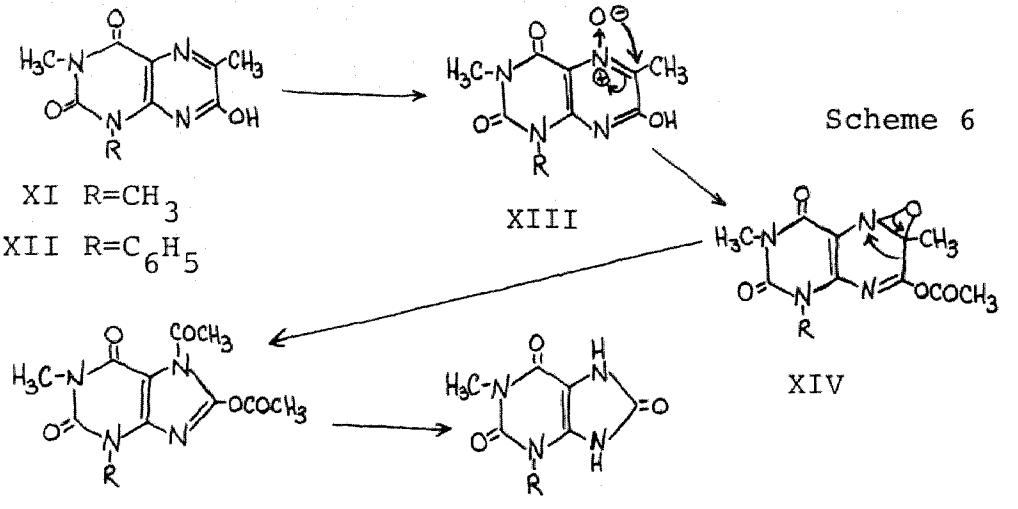
Bagnara (7) questions this scheme since pteridines and purines are not located in the same chromatophore rendering it difficult to envision that a precursor at as high a level as guanylic acid could be a key in determining whether either purine or pteridine synthesis is to be favored. Alternately, Bagnara proposes that purine and

[1]Hypophysioprivic refers to an organism with the pituitary gland removed or deactivated.

pteridine production are governed by the action of the hormone, intermedin, acting directly on the iridophore. If the increased purine content of iridophores is a function of long-term chromatophore expansion, then intermedin could cause concentration of pigment granules in the iridophores. This would render synthetic sites on the purine granules less accessible. Elementary precursors which are common to both pteridine and purine biosynthesis are then not tied up in the iridophore and instead may diffuse out to become available to the pteridine biosynthetic system on the xanthophore. A decrease in purine synthesis and an increase in pteridine production is the net result. However, when intermedin is lacking, iridophore pigments are dispersed and purine synthesis would be favored at the expense of pteridine production. (7)

Chemical and biological conversion of pteridines to purines. While the details of guanine synthesis via normal metabolic routes are well known (40,41,44,45), it is only recently that pteridine to purine conversions have been reported. Hutzenlaub, Barlin, and Pfleiderer (34) have described the chemical transformation of 7-hydroxy-1,3,6-trimethyl-2,4-dioxotetrahydropteridine (Scheme 6, XI) and the 3,6-dimethyl-1-phenyl derivative (XII) to give the ring contraction and purine products, 1,3-dimethyluric acid (XVI) and 1-methyl-3-phenyluric acid (XVII), respectively, shown in Scheme 6. This ring contraction of the pyrazine to the

imidazole structure has been observed in vivo only in the bacterial organism, Alcaligenes faecalis. (34)



Scheme 6

Although it appears that pteridine to purine conversions are not common in organisms, such an abnormality may occur. This abnormality may be hormonal, as outlined by Bagnara (7), or it may be enzymic, with the specific enzymes heretofore unidentified. (37) Either may possibly be the cause of the opaque mutation in Bettas.

Examine now, the pigmentary relationships between the various Betta mutant types used in this research. All the phenotypes used appeared to have pteridines present. (26) However, the opaque whites apparently lack red pteridines. The Op mutants were developed from a pastel stock, the pastels originating as a genetic variety of yellows. (1, 2,46) These mutants, their genotypes, and characteristic

differences from one to the next are described in Appendix G. From the yellow to opaque white phenotype, the fish loses the ability to make colored pigments (pteridines) and increases its ability to form and/or accumulate the purine, guanine.

As previously discussed, Op does occur in Bettas other than the white and pastel mutants. In light of this, an interesting color combination, seemingly unrelated to Op, may bear further consideration in terms of chemical analyses and genetic tests. The highly variable Si mutation may provide some clues about the iridophore-pteridine interrelationship:

> The fish may be nearly covered with sheen or it may be spread, but with "thin" places on various parts of the fins. The very dense coating may almost completely cover color that lies underneath, such as red. If so, it may be difficult to see what red the fish actually has. The "thin" places, especially the fins, may allow red to show through so that the fins are often red-green, red-blue, green-red or blue-red. The color appears to blend as a wash rather than to be sharply defined as is the red when variegated. (29)

This description of a Betta with heavy pteridine and iridophore pigmentation may indicate that red pteridine synthesis is decreased where iridophore pigment synthesis is increased. It is possible that red color is not covered, rather its production is simply lessened in favor of the purine cover. In "thin" places, less purines may be produced and more pteridines observed. As this discussion

indicates, several genetic crosses with subsequent chemical analyses of the hybrids are needed to unravel the genetic and chemical nature of the Op mutation.

Guanine Gout. Another explanation of the guanine accumulation in Op Bettas may be related to faulty purine metabolism. A summary of normal nitrogen excretion in fishes (47) shows that urine accounts for 2.5-24.5% of the total nitrogen excreted. The nitrogen-containing components in urine include creatine, urea, ammonia, amino acids, and uric acid. The gills are the principle route for removal of ammonia and urea, the major nitrogeneous wastes of fishes. Aquatic ammonotelism is the major process of nitrogen removal for fresh water and marine teleosts. (4) Guanotelism (excretion of guanine) occurs in some organisms such as scorpions, spiders, and certain birds. (48) In other animals, excess quantities of nitrogeneous materials not normally excreted may be converted to harmless by-products and stored in the body integuments. (47)

Invertebrates accumulate high concentrations of purines in their tissues for subsequent metabolic use. This may, however, reach pathological proportions. (48) In man the over-production of uric acid, occurring principally in males, results in the disease gout. Elevated levels of uric acid are detected in the blood and body fluids. Excess quantities form the rather insoluble salt, sodium urate, and are deposited in crystalline form in the cartilage. This is

actually a type of arthritis and causes extreme sensitivity of the joints. (41,44,49) Pigs have been found to suffer from guanine gout in a manner comparable to the disease of man. (50)

The normal catabolism of purines to uric acid, allantoin, and urea is shown in Figure 18. A parallelism has been drawn between the absence of uricase in man and the absence of guanase in the pig. (50) The lack of enzyme activity would prevent the substrate from being converted to the next product in the sequence. Current reviews indicate that enzyme abnormalities involving glutathione reductase, xanthine oxidase, glutaminase, or glutamate dehydrogenase may cause primary gout in man. (51) A simple comparison illustrates parallel enzymic abnormalities in man and the pig. If uricase is inactive in man, uric acid is not converted to allantoin and hence to urea; uric acid accumulates and is stored in the joints of the body. Similarly, guanase inactivity in the pig prevents guanine conversion to xanthine and then to uric acid; guanine accumulates and is stored in the pig's body integuments. This shows the occurrence of high uric acid (or guanine) concentrations in the body as a result of reduced catabolism. Overproduction, lowered excretion, or any combination of these three may also result in purine accumulation. (51,52)

Another example of enzymic guanine accumulation is observed in goldfish. Iridophore degeneration of the

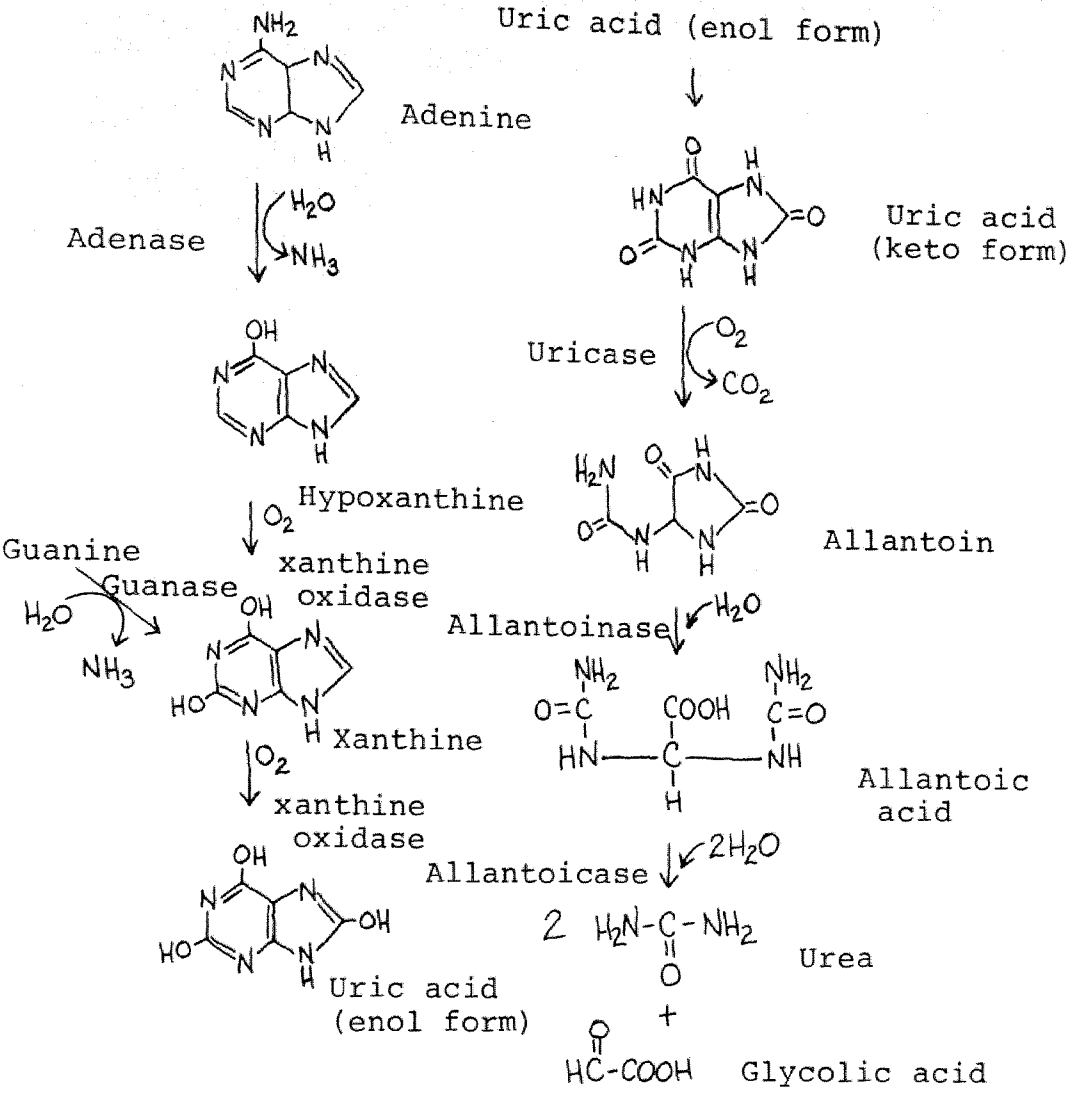


Figure 18. Degradation of purines to uric acid, allantoin, and urea. (23)

transparent-scaled goldfish occurs about 10 days after hatching when embryos are cultured in an enzyme inhibiting media. Phenylthiourea inhibitor causes lack of activity for both tyrosinase and Dopa-oxidase. Neither pituitary nor thyroid gland were active in this manifestation of gene

action. (4) Iridophore degeneration is accompanied by an increase in guanine deaminase activity suggesting its participation in larval iridophore decomposition of guanine granules.

Guanine accumulation in the opaque white Bettas may be a form of guanine gout similar to that described for the pig. If guanase were inactive or absent, guanine would not be converted to normal by-products (Figure 18). Note also in this scheme that hypoxanthine results from adenine catabolism. Small quanitites could build-up if the turnover action of xanthine oxidase was appreciably slowed as guanine is not converted to xanthine. Consequently, the rate of adenine catabolism may be appreciably slower than that of guanine catabolism.

Suggestions for Further Study. There are several experiments that should be done to elucidate the process resulting in the Op mutation. For existing Betta phenotypes a normal, Si, and Op would have to be compared.

The interrelationship between pigmentary materials of chromatophores might be studied in several ways. Pteridines in the opaque white Bettas and in other phenotypes with the Op mutation, both homozygous and heterozygous, should be identified. Carotenoids may also be present as xanthophore pigments. (26) Correlation of these pigments to the purines present may suggest where conversions are abnormal in the pigmentary system.

Blood levels of the hormone, intermedin, should be determined in Bettas with the Op mutation and correlated to a decrease in pteridines. The specific enzyme activity and serum (or organ) level of guanase should also be determined. These tests may indicate whether the abnormality is hormonal or enzymic.

The physical character of opaque material purines should be compared to normal iridophore material and purines in fish with the Si mutation. In general, granular guanine particles are mobile in cell cytoplasm while platelet guanine occurs as relatively immobile stacks. (4) Microscopic studies are needed to determine if the guanine in Op Bettas exists as material stored free in body integuments or is associated with iridophores, and, if so, in what form.

Physical and chemical analyses of genetic hybrids are also needed. Thus far, the Op mutation occurs only in fish with the Si mutation also. It has not yet been determined whether the Op mutation can occur alone or if an intermediate genetic expression occurs when the Si mutation is absent. Quantitative measures of pteridines in fish identical except for Op might provide information for a pigmentary interrelationship. Clearer comparisons can be made from identical phenotypes with variation in only a single mutation factor. In practice, this may be a difficult and time-consuming goal.

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APPENDICES

APPENDIX A. *Betta* Genetics

Mutations are symbolized by using upper and lower case letters indicating dominance and recessiveness relative to the normal or wild-type state of pigmentation. The symbols used in genetic notation of genotype are summarized as follows:

+//+	Normal genotype
mutation//mutation	Homozygous
mutation//+	Heterozygous
mutation//?	Complete genotype not known

Mutations are described by comparison of the mutant to the wild Bettas from Thialand, *Betta splendens* (Figure 5). The genotypes for the fish used in this study are given below.

Phenotype	Opaque Mutation (Op)	Spread Iridocytes Mutation (Si)
Normal	+//+	+//+ (minimal Si)
Spread iridocytes	+//+	Si//?
Opaque white	Op//Op	Si//?
Hybrids (Op x Si)	Op//+	Si//?

APPENDIX B. R_f Values for Thin Layer Chromatography

G = Guanine
H = Hypoxanthine
P = Pteridines

Phenotype	Fins	Body	Head
Opaque white			
1	G:0.28 H:0.48 P:0.00	G:0.26 H:0.44 P:0.00	G:0.29 H:0.51 P:0.00
2	G:0.29 H:0.51 P:0.00	G:0.26 H:0.46 P:0.00	G:0.28 H:0.48 P:0.00
3	G:0.30 H:0.52 P:0.00	G:0.29 H:0.50 P:0.00	G:0.30 H:0.53 P:0.00
4	G:0.30 H:0.50 P:0.00	G:0.29 H:0.49 P:0.00	G:0.29 H:0.48 P:0.00
5	G:0.28 H:0.50 P:0.00	G:0.29 H:0.49 P:0.00	G:0.30 H:0.50 P:0.00
6	G:0.29 H:0.48 P:0.00	G:0.28 H:0.48 P:0.00	G:0.27 H:0.49 P:0.00
7	G:0.30 H:0.50 P:0.00	G:0.28 H:0.47 P:0.00	G:0.28 H:0.48 P:0.00
Normal			
1	G: H:0.52 P:0.00	G:0.30 H:0.51 P:0.00	G:0.37 H: P:0.00
2	G:0.33 H:0.53 P:0.00	G:0.32 H:0.49 P:0.00, 0.63	G:0.38 H: P:0.00

Phenotype	Fins	Body	Head
Normal (cont.)			
3	G:0.30 H:0.51 P:0.00, 0.06, 0.20, 0.64	G:0.30 H:0.48 P:0.00, 0.63	G:0.30 H:0.52 P:0.00,0.68
	All spots fluoresced indicating the possible presence of pteridines.		
Spread iridocytes			
1	G:0.29 H:0.50 P:0.00, 0.20, 0.42, 0.60	G:0.29 H:0.65 P:0.00, 0.11, 0.58	G:0.27 H:0.62 P:0.00,0.15, 0.60
2	G:0.30 H:0.51 P:0.00, 0.14, 0.26, 0.60	G:0.30 H:0.63 P:0.00, 0.12, 0.59	G:0.30 H: P:0.00,0.15, 0.62
3	G:0.25 H:0.44 P:0.00, 0.18, 0.60	G:0.26 H:0.43 P:0.00, 0.12, 0.60	G:0.25 H:0.49 P:0.00,0.14, 0.62
	All spots fluoresced indicating the possible presence of pteridines.		
Hybrid			
1	G:0.29 H:0.48 P:	G:0.29 H:0.51 P:	G:0.30 H:0.52 P:
	Some hypoxanthine spots fluoresced slightly indicating the possible presence of pteridines.		
2	G:0.30 H:0.52 P:0.00	G:0.28 H:0.49 P:0.00, 0.61	G:0.30 H: P:0.00,0.65
3	G:0.28 H:0.47 P:0.00, 0.10, 0.20, 0.38, 0.59	G:0.31 H:0.47 P:0.00, 0.16	G:0.30 H:0.53 P:0.00,0.14

Standards

Guanine	Hypoxanthine
0.31	0.58
0.32	0.56
0.34	0.56
0.31	0.57
0.33	0.57
0.34	0.56
0.33	0.55
0.33	0.57
0.31	0.57
0.34	0.56
0.33	0.55

APPENDIX C. Ultraviolet spectra - Absorption maxima

Phenotype	GUANINE λ_{max}	GUANINE Shoulder	HYPOXANTHINE λ_{max}
Opaque white			
1	250	274	250
	247	273	250
	246	272	250
2	248	274	250
	247	273	250
	250	273	250
3	246	273	245
	245	273	244
	245	273	244
4	247	273	245
	248	273	245
	248	273	245
5	252	273	255
	252	273	255
	253	274	255
6	248	273	255
	250	273	250
	249	273	251
7	251	273	253
	251	273	255
	249	274	255
Normal			
1			
2			
3	245		243
	243		244
	243		244

Phenotype	GUANINE λ_{max}	GUANINE Shoulder	HYPOXANTHINE λ_{max}
Spread iridocytes			
1	247	275	
	248	273	
	248	274	
2	250	273	
	250	273	
	247	273	
3	246	272	
	248	273	243
	248	273	243
Hybrid			
1	248	273	248
	250	274	248
	249	274	248
2	247	274	
	248	273	
	250	273	
3	245	273	243
	247	273	243
	248	273	243
Standards			
	249	273	248
	248	273	247
	246	273	249
	248	273	248
	248	273	247
	247	273	248
	247	273	248
	248	273	247

Pteridines

Component extracts of sample spots and fronts from the TLC plates showed tailing from about 240 nm to lower wavelengths. This tailing also appeared for several of the guanine and hypoxanthine component extracts from the various fish phenotypes. Such tailing was not observed for component extracts of sample spots and fronts taken from the TLC plates of standard guanine and hypoxanthine solutions.

APPENDIX D. Fluorescence spectra - Emission maxima

	Maxima - Wavelength(Intensity)		
Reference (0.1 N HCl)	256(13)	378(6)	496(62)
	257(15)	378(5)	498(60)
	256(13)	392(5)	497(61)
	256(12.5)	396(5)	496(60)
	256(9)	412(6)	496(62)
	258(15)		500(32)
	256(14)	420(10)	498(64)
	257(14)	415(10)	498(64)
	256(13)		500(48)
	257(12)		498(61)
	257(12)		498(54)
	258(9)		500(48)
	258(18)		500(42)
	257(11)	400(7)	
	256(17)	380(12)	
	256(14)	393(6)	497(59)
	257(13)		498(69)
	256(9)	372(6)	498(61)
	258(13)		498(68)
	257(12.5)		500(48)
	256(14)	404(12)	497(63)
	258(13)		498(68)
Phenotype			
Opaque white	(Reference)	358(41)	680(4.5)
	"	356(58)	668(9)
	"	358(92)	679(21)
	"	354(68)	673(12)
	"	358(48)	668(7)
	"	356(69)	669(13)
	"	356(52)	664(4)
	"	358(49)	678(11)
Normal	(Reference)	358(19)	
	"	358(13)	
Spread iridocytes	(Reference)	356(10.5)	
	"	358(21)	
	"	356(21)	595(8)
Hybrid	(Reference)	354(35.5)	587(7)
	"	312(16)	592(9)
	"	348(19)	

		Maxima - Wavelength(Intensity)			
Standards					
Guanine	(Reference)	358(36)		679(5)	
	"	357(67.5)		672(10)	
	"	357(61.5)		667(9.5)	
Hypoxanthine	(Reference)				
	"				
	"				
Pteridines	(Reference)	348(20)	420(270)		625(5)
	"	338(24)	418(27)		648(7)
	"	338(24)	418(25)	540(13)	648(7)
	"	305(11)		587(6)	
	"	358(9)			

APPENDIX E. Quantitative analysis - Weights of integuments

Phenotype	Fins	Body	Head
Opaque white	3.165 mg	25.900 mg	3.530 mg
Normal	5.865	12.540	13.100
Spread iridocytes	22.910	36.300	14.860
Hybrid	13.060	27.970	9.825

APPENDIX F. Ultraviolet spectra - Uric acid, urea, allantoin

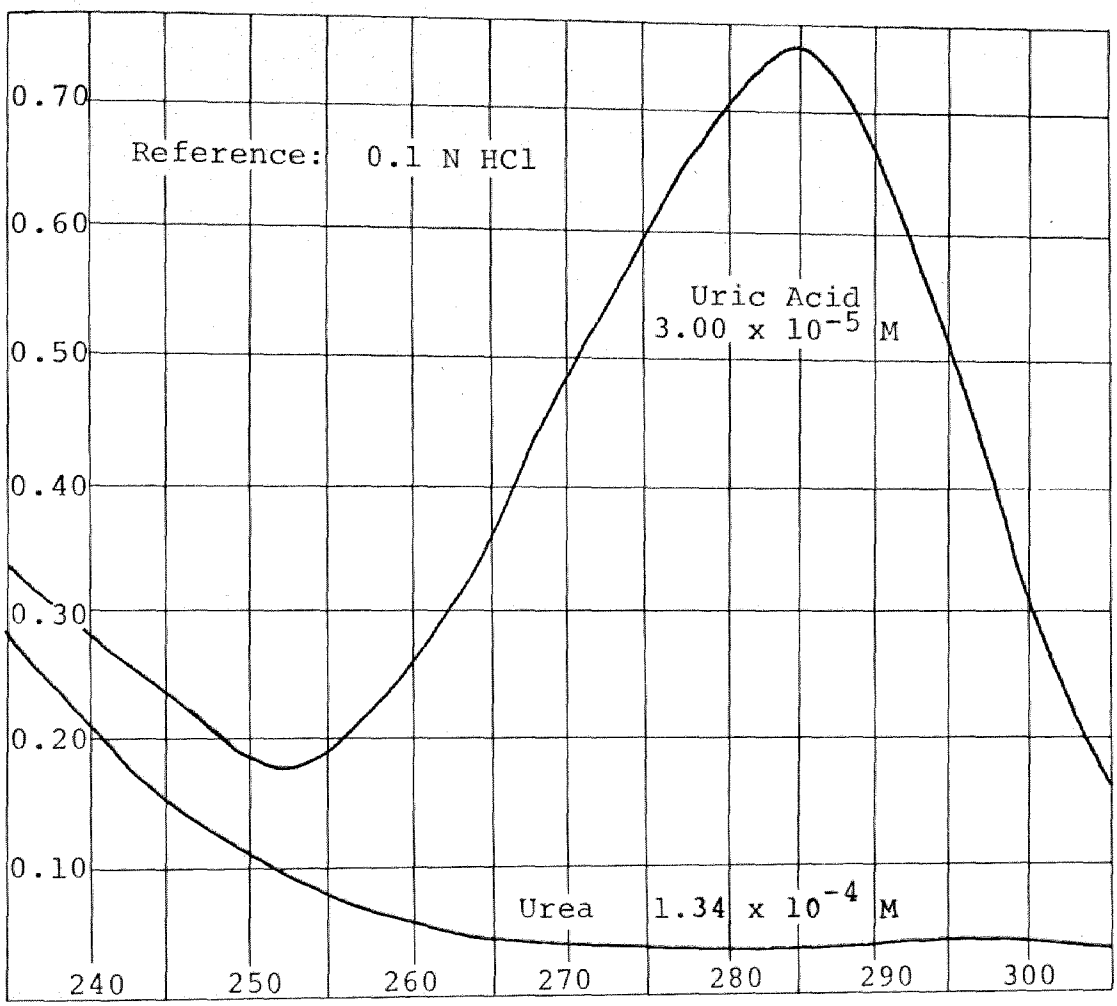

Figure 19. Typical ultraviolet spectra of uric acid and urea.

Table 13. Optical properties for uric acid, urea and allantoin.

Compound	λ_{max}	ϵ
Uric acid	284 nm	12.0×10^3
Urea	225 nm	1.24×10^3
Allantoin	224 nm	17.7×10^3

APPENDIX G. Genotype and description of Betta mutants from yellow to opaque white

Mutant	Genotype	Description
Yellow	R//?, nr//nr, c//c	R//? is extended red. If able to make red pigment it would be over the entire body and fins. nr//nr is non-red. The fish cannot produce red pigment and is thus yellow where it normally would be red. c//c is cambodia. The fish cannot produce the black pigment, melanin.
Pastel	R//?, nr//nr, c//c, Si//?, (Bl//+, Bl//Bl, or +//+), Op//?	Si//? is spread iridocytes. These fish are like the yellows except that the iridophore color or metallic sheen is spread over the entire body and fins, with only limited amounts on the head. +//+ is normal, Green color. Bl//+ is Blue coloration. Bl//Bl is Steel Blue color. The Bl gene (=Blue) removes or reduces the fishes' ability to make yellow pigment. An intermediate effect occurs, thus three phenotypes are observed. Op//? is opaque. The fishes' general appearance is opaque or creamy, especially on the head. Homozygous fish are probably more affected.

Mutant	Genotype	Description
Opaque White	R//?, nr//nr, c//c, Si//?, Bl//Bl, Op//Op	These fish cannot make red or black pigments and have iridophores over the entire body. The steel blue mutation appears to remove or reduce the fishes' ability to make yellow pigments. Their general appearance is opaque or creamy, especially on the head where metallic sheen does not occur. The opaque material is also deposited on the cornea, both inside and outside. The material accumulates as the fish ages.

APPENDIX H. R_f values from thin layer chromatography comparison of guanine, 1-methylguanine, and hypoxanthine with extracted material from Op Bettas.

Plate: Cellulose with fluorescent indicator (Eastman Prepared Chromagrams)
Solvent: 1M NaCl, aqueous
Visualization: Short wavelength U.V. light
Temperature: Ambient

Plate 1. Doubled concentration of 1-methylguanine on each spot.

	Guanine	1-Methylguanine[1]		
	0.41	0.41	0.46	0.62
	0.39	0.41	0.42	0.60
	0.38	0.38	0.43	0.59
	0.41	0.41	0.47	0.61
Average:	0.40	0.40	0.44	0.60

Plate 2,3.

Guanine	1-Methylguanine[1]		Hypoxanthine	Op Fish
0.41	0.40	0.62	0.60	0.39
0.40	0.39	0.62	0.59	0.39
0.36	0.37	0.57	0.58	0.34
Average:				
0.39	0.39	0.60	0.59	0.37

[1] ICN Life Science Group, Cleveland, Ohio.

Plate 4.

Plate: Cellulose with fluorescent indicator (Eastman Prepared Chromagrams)
Solvent: 1-Butanol, water, 98% formic acid (77:13:10)[2]
Visualization: Short wavelength U.V. light
Temperature: Ambient

	Guanine	1-Methylguanine[1]		Hypoxanthine	Op Fish
	0.23	0.22	0.37	0.36	0.22
	0.23	0.22	0.35	0.33	0.21
					0.22
Average:	0.23	0.22	0.36	0.34	0.22

[1] ICN Life Science Group, Cleveland, Ohio.

[2] Taken from reference 53.